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## EFFECT OF CERTAIN GRAIN RATIONS ON THE GROWTH OF THE WHITE LEGHORN CHICK

By G. DAVIS BUCKNER, E. H. NOLLAU, R. H. WILKINS, and JOSEPH H. KASTLE

*Department of Chemistry, Kentucky Agricultural Experiment Station*

In a former paper<sup>1</sup> from this laboratory evidence was presented to show that the lysin content of the proteins of certain grain mixtures fed to White Leghorn chicks was the limiting factor in their growth. The results of those experiments showed that on a ration consisting of wheat, wheat bran, sunflower seed, hempseed, cracked corn, skim milk, cabbage, and sprouted oats, normal growth was obtained, while on a ration consisting of barley, rice, hominy, oats, gluten flour, butter fat, cabbage, and sprouted oats a condition of arrested growth resulted. The first-mentioned ration was supposed to contain a high percentage of the amino acid, lysin, as compared with the second ration, which was supposed to be low in lysin. These experiments were open to criticism because of the small number of chicks under consideration, the laboratory conditions governing them and the possible inaccuracy in the numbers given for the amino-acid distribution of the grain mixtures fed. In view of this, an experiment was planned which would, as far as possible, eliminate these points of objection.

In 1915 an experiment was conducted by Buckner, Nollau, and Kastle, in which 14 one-day-old White Leghorn chicks were fed a ration consisting of 33 parts of ground soybeans and 67 parts of ground oats, supplemented by 20 per cent of protein-free milk, sprouted oats, shredded cabbage leaves, grit, oyster shell, and a small quantity of sour skim milk. On this ration the chicks failed to thrive and grow and would eventually have died had not the grain ration been changed. It was changed to equal parts of wheat bran, sunflower seed, hempseed, barley, oats, and rice. On this diet a partial recovery was effected, yet the vigor and development of the normal White Leghorn chick of a similar age was not attained. One of us (Kastle) contended that this failure to grow

<sup>1</sup> BUCKNER, G. D., NOLLAU, E. H., and KASTLE, J. H. THE FEEDING OF YOUNG CHICKS ON GRAIN MIXTURES OF HIGH AND LOW LYSIN CONTENT. *In Amer. Jour. Physiol.*, v. 39, no. 2, p. 162-171, 1 pl. 1915.

normally was caused by some toxic principle in the soybean, and in order to throw more light on this point the following experiment was planned. It was conducted in an airy, well-lighted basement room of the Kentucky Agricultural Experiment Station.

Two lots of chicks, designated "A" and "B," consisting of 12 and 11 White Leghorn chicks, respectively, were selected entirely at random and fed rations composed of equal parts of soybeans, oats, wheat, ship stuff, sunflower seed, and cracked corn, supplemented by sour skim milk, sprouted oats, shredded cabbage, grit, and oyster shell. The food of lot A was given to them in the usual manner of feeding. In the case of lot B the grains were ground together, well mixed with a small quantity of distilled water, and baked in an electric oven. The temperature of the oven was kept at 420° F., and the mixture was frequently stirred. The baked feed was ground and fed to lot B in the same way as was the uncooked mixture to lot A. Table I gives the weights of the chicks, their increase in weight, and the number surviving each week.

TABLE I.—Effect of uncooked and cooked rations on the growth of White Leghorn chicks

LOT A						
Date.	Number of weeks.	Number of chicks.	Total weight of chicks.	Average weight of chicks.	Average increase.	Average percent-age increase.
1915.						
July 6.....		11	Gm. 528	Gm. 48.0	Gm.	
13.....	1	11	725	65.9	17.9	37.3
20.....	2	11	1,004	91.3	25.4	38.5
27.....	3	11	1,266	115.1	23.8	26.1
Aug. 3.....	4	10	1,832	183.2	68.1	59.1
10.....	5	10	2,462	246.2	63.0	34.3
18.....	6	10	3,290	329.0	82.8	33.6
24.....	7	10	3,795	379.5	50.5	15.4
31.....	8	10	4,357	435.7	56.2	14.8
Sept. 7.....	9	9	4,410	490.0	54.3	12.4
LOT B						
1915.						
July 6.....		12	554	46.2		
13.....	1	12	788	65.7	19.5	42.2
20.....	2	11	1,014	92.2	26.5	40.3
27.....	3	8	930	116.2	24.0	26.0
Aug. 3.....	4	8	1,357	169.7	53.5	46.4
10.....	5	7	1,655	236.4	66.7	39.3
18.....	6	7	2,160	308.5	72.1	30.4
24.....	7	7	2,307	329.5	21.0	6.8
31.....	8	4	2,062	515.5	186.0	50.4
Sept. 7.....	9	4	2,275	568.2	50.2	9.8

\* Three chickens sick.

This experiment, as shown in Table 1, covers a period of nine weeks in which both lots of chicks received identical treatment, except as to the preparation of the food. The growth of lot A was approximately normal as to weight and mortality, but their vigor and general condition was not good. In lot B, which received the cooked grain ration, a decided deleterious effect was shown by the weight and mortality record which can properly be ascribed to the ration. If we regard this ration in the light of the present-day conception of nutrition, it is balanced with reference to the dietary essentials, fat-soluble A being abundantly supplied in the sprouted oats, shredded cabbage leaves, and butter fat contained in the not-too-closely skimmed milk, while sufficient water soluble B was obtained from the grain mixture before them as a dry mash at all times. The incomplete proteins of the grains were supplemented by the casein of the milk in the wet mash and the proteins in the cabbage and sprouted oats. These factors, with the mineral content amply supplied by grit, oyster shell, and milk, satisfy all conditions in making a complete diet. However, the food hormones were destroyed by heating the ration fed to lot B.

The experiments of McCollum and his coworkers have demonstrated the nutritive limits of seeds. McCollum states<sup>1</sup> in substance that when seeds are fed, supplemented by suitable inorganic salts and sufficient fat-soluble A, the limiting factor with respect to growth is the quality of protein. The data presented in this paper and the experimental work to be described corroborate this statement and throw further light on the following points:

1. That the soybean may enter into the dietary of the White Leghorn chick without having an accumulative deleterious effect.
2. The effect of heat on the food value of certain grain mixtures.
3. That under approximately ideal conditions chicks which had been stunted by dietary measures and had survived by reason of greater vitality may remain in a fairly good state of health over a long period of time.

The plan of the experiments was as follows: Four grain rations were selected so that two would contain grains the proteins of which were supposed to be high in lysin (one to be fed as a mash and one as a grain); the other two grain mixtures were supposed to contain proteins low in lysin and were to be fed in the same way as the first two. After this experiment had been started an effort was made to determine the amino-nitrogen distribution in these mixtures, but, owing to their large carbohydrate content, no satisfactory results have been obtained. The effort to analyze these complex grain mixtures is still in progress<sup>2</sup> and it is

<sup>1</sup> McCOLLUM, E. V., and SIMMONDS, N. A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS. III. THE VALUE OF SOME SEED PROTEINS FOR MAINTENANCE. *In Jour. Biol. Chem.*, V 37, No. 3, p. 347-508, 12 charts. 1917.

hoped that their amino-acid make-up may be determined at some future time.

In this experiment we selected at random from 600 1-day-old incubator chicks of the White Leghorn breed, three lots, each containing 60 chicks, which were kept under identical conditions except that the diets were different. The conditions governing these three lots of chicks were as follows:

Lot 1 was placed in a large brooder house which opened on a large grass run. The chicks were weighed individually on a torsion balance, sensitive to 0.1 gm. the day after they were hatched and were so weighed each succeeding week. The weekly individual weighings and mortality records were kept, the previous weight records of all chicks which had died being discarded, so that at the end of 28 weeks the weight records represented only those that were living at that time. No particular care was taken of these chicks except to see that they were properly watered, fed, and housed. They were fed a ration consisting of equal parts of finely ground soybeans, wheat, wheat bran, sunflower seed, and hempseed. This was fed as a dry mash and was kept before them at all times; and once a day, at noon, a wet mash of this mixture made with sour skim milk was fed. Once a day a coarsely ground grain mixture, consisting of equal parts of wheat, soybeans, hempseed, and cracked corn, was thrown into the litter in order to make them exercise by scratching for it. Oyster shell, grit, and charcoal were before them at all times, while sprouted oats and shredded cabbage leaves were liberally fed once a day. It may be of interest to note that this ration is in every way identical, with the exception that soybeans were added in an equal quantity, with the ration fed to lot 3 of our previous paper,<sup>1</sup> in which the growth and general physical condition were normal, even though the experiment was conducted under laboratory conditions.

These chicks were closely watched, and any change in their physical condition and habits was noted. When the treading period began, the cockerels and pullets were separated to avoid any loss of weight occasioned by undue exercise.

Lot 2 was kept under the same conditions, only this lot could not be separated into cockerels and pullets, because their external sexual characteristics were depressed to such an extent that they could not be distinguished, and treading did not occur. A dry mash, which was kept before them at all times, consisted of equal parts of finely ground barley, rice, hominy, and oats, to which was added enough gluten flour and butter fat to raise the protein and fat content of this grain mixture up to that of the grain mixture fed as a mash to lot 1. Once a day they received a wet mash made of this grain mixture moistened with protein-free milk. The grain ration fed in the litter consisted of equal parts of

<sup>1</sup> BUCKNER, C. D., NOLLAU, E. H., and KASTLE, J. H. 1915. *OP. CIT.*

barley, rice, and hominy, to which was added enough pure butter fat and gluten flour to bring the fat and protein content up to the same level as that of the corresponding grain mixture fed to lot 1.<sup>1</sup>

Lot 3 constituted the control for the two other lots of chicks. This lot was kept under conditions identical with those of lots 1 and 2, the cockerels and pullets being separated when treading began. These chicks received a ration known as the standard Cornell ration.<sup>2</sup>

Table II presents the weekly weight and mortality records of these three lots of chicks, covering a period of 28 weeks, in which time there were no unusual weather conditions or epidemics among these chicks, so that these figures represent the degree of nourishment afforded by the rations fed to the separate lots.

TABLE III.—Effect of various diets on the growth of White Leghorn chicks

Week.	Lot 1.			Number of chicks	Lot 2.			Number of chicks	Lot 3 (control)			Number of chicks
	Average weight of—				Average weight of—				Average weight of—			
	25 cocks.	17 hens.	Mean.		25 cocks.	17 hens.	Mean.		25 cocks.	17 hens.	Mean.	
	Gm.	Gm.	Gm.		Gm.	Gm.	Gm.		Gm.	Gm.	Gm.	
0.....	41.0	39.4	40.2	50	41.8	39.1	40.4	50	42.1	41.1	41.6	50
1.....	70.4	66.5	68.4	50	62.2	59.1	60.6	50	72.2	69.1	70.6	50
2.....	100.9	96.8	98.8	55	86.1	84.	85.0	55	109.7	97.6	103.6	55
3.....	146.5	138.7	142.6	55	126.1	122.	124.0	55	149.9	140.9	145.4	55
4.....	184.7	179.1	181.9	55	169.7	165.	167.4	55	191.7	181.7	186.7	55
5.....	216.5	216.5	216.5	55	208.8	202.8	205.8	55	228.8	218.8	223.8	55
6.....	256.5	256.5	256.5	55	246.9	236.9	241.9	55	268.9	258.9	263.9	55
7.....	303.4	318.5	310.9	55	284.1	274.1	279.1	55	318.1	308.1	313.1	55
8.....	420.6	431.5	426.0	55	398.8	388.8	393.8	55	438.8	428.8	433.8	55
9.....	474.0	481.5	477.7	55	448.6	438.6	443.6	55	488.6	478.6	483.6	55
10.....	518.2	528.2	523.2	49	498.4	488.4	493.4	49	538.4	528.4	533.4	49
11.....	568.5	578.5	573.5	48	548.1	538.1	543.1	48	588.1	578.1	583.1	48
12.....	613.4	623.4	618.4	48	593.1	583.1	588.1	48	633.1	623.1	628.1	48
13.....	713.4	723.4	718.4	45	693.1	683.1	688.1	45	733.1	723.1	728.1	45
14.....	795.8	805.8	800.8	45	773.1	763.1	768.1	45	813.1	803.1	808.1	45
15.....	868.4	878.4	873.4	45	853.1	843.1	848.1	45	893.1	883.1	888.1	45
16.....	914.0	924.0	919.0	45	903.1	893.1	898.1	45	943.1	933.1	938.1	45
17.....	1,014.5	1,024.5	1,019.5	45	1,003.1	993.1	998.1	45	1,043.1	1,033.1	1,038.1	45
18.....	1,077.8	1,087.8	1,082.8	45	1,053.1	1,043.1	1,048.1	45	1,093.1	1,083.1	1,088.1	45
19.....	1,115.9	1,125.9	1,120.9	44	1,093.1	1,083.1	1,088.1	44	1,133.1	1,123.1	1,128.1	44
20.....	1,154.0	1,164.0	1,159.0	44	1,133.1	1,123.1	1,128.1	44	1,173.1	1,163.1	1,168.1	44
21.....	1,214.5	1,224.5	1,219.5	44	1,173.1	1,163.1	1,168.1	44	1,213.1	1,203.1	1,208.1	44
22.....	1,214.5	1,224.5	1,219.5	44	1,173.1	1,163.1	1,168.1	44	1,213.1	1,203.1	1,208.1	44
23.....	1,214.5	1,224.5	1,219.5	44	1,173.1	1,163.1	1,168.1	44	1,213.1	1,203.1	1,208.1	44
24.....	1,214.5	1,224.5	1,219.5	44	1,173.1	1,163.1	1,168.1	44	1,213.1	1,203.1	1,208.1	44
25.....	1,250.6	1,260.6	1,255.6	41	1,213.1	1,203.1	1,208.1	41	1,253.1	1,243.1	1,248.1	41
26.....	1,250.6	1,260.6	1,255.6	41	1,213.1	1,203.1	1,208.1	41	1,253.1	1,243.1	1,248.1	41
27.....	1,250.6	1,260.6	1,255.6	41	1,213.1	1,203.1	1,208.1	41	1,253.1	1,243.1	1,248.1	41
28.....	1,254.3	1,264.3	1,259.3	41	1,213.1	1,203.1	1,208.1	41	1,253.1	1,243.1	1,248.1	41

In studying the growth record of lot 1 it will be seen that the average weights of the pullets closely followed the control throughout the 28 weeks, but that the cockerels were inferior in this respect after the third

<sup>1</sup> At the expiration of these 28 weeks the ration was changed only by the addition of sour skim milk, which brought about profound anatomical and physiological changes. The combs and wattles developed, shortly to be followed by crowing and treading on the part of the cockerels. It is also of interest to note that their growth was greatly accelerated. The exact figures could not be counted on because of errors occasioned by change of student assistants helping in the weighing.

<sup>2</sup> NIXON, Clara. FEEDING YOUNG CHICKENS. N. Y. State Col. Agr. Cornell Reading Course, v. 4, no. 88, p. 176. 1915.

week. Regarding their physical condition after the sixth week it was plainly to be seen that lot 1 did not have the same vigorous appearance as lot 3. This was most particularly noticed with regard to the general condition of the feathering which seemed slightly ruffled and unkempt as compared to lot 3. This can be seen only to a degree in the illustrations of these two lots (Pl. 42 A, B). Their general vigor and development did not appear equal to that of lot 3, and these differences became more noticeable as they grew older, until, at the end of the twenty-eighth week, when the experiment was discontinued, it was plainly seen that lot 1 was in no way in the same physiological state as lot 3.

It will also be noted from the data that after the seventeenth week lot 1 did not gain as much as lot 3 over the same period. During these 11 weeks the gain was 234.2 gms. as against 286.5 gms. for lot 3; or, in other words, lot 3 made a gain 18.3 per cent greater than that made by lot 1. It may be of interest to point out that the cockerels of lot 1 showed a greater variation in weight from the normal than the pullets. The results of the mortality record show the same number of deaths at the end of the experiment as in the control.

In lot 2 we see that the rate of growth was retarded to such an extent that at the end of the 28 weeks the average weight of a chick was 809.4 gms. as against 1,238.4 gm. for those receiving the ration containing soybeans and 1,357.5 gms. for the control. The external sexual characteristics were rudimentary, and the feather tracts not properly developed and the fact that they retained the habits of the immature chick during this entire period is of added interest. This is shown in the illustration of this lot (Pl. 42, C) which was taken the same day as those of lots 1 and 3, all having the same focal distance. The ration fed to lot 2 greatly increased the mortality so that only 19 remained at the end of the experiment, the weaklings having died earliest. Therefore we had only those with the greatest initial vitality which had lived for 28 weeks on a diet the biologic value of which was low. From this we would infer that the individual vitality of the animal plays a very important part in determining its ability to grow, and for this reason it is essential that a considerable number of animals should be used in experiments on nutrition. This would seem to follow when we realize that all of the chicks used in these experiments were pure-bred and came from the same parent stock. The chicks of lot 2 seemed to have a good appetite, and while they ate with apparent avidity, yet they always seemed to be in search of something in their feed which they could not find. Regarding their ration, it will be seen that it is satisfactory with respect to every necessary dietary factor except the quality of protein. We are unable at this time to show wherein these proteins are limited, but hope to be able to prove this by experiments which are now in progress.

In conclusion we feel justified in making the following deductions from the results of these experiments:

(1) The proteins of rice, oats, barley, hominy, and gluten flour are inefficient in promoting normal growth in the White Leghorn chick.

(2) The results of these experiments seem to indicate that the proteins in the grains mentioned above have a retarding action on the development of the external sexual characteristics and their functions, which accompanies the arrested growth of the chicks.

(3) We are unable to account for the apparent weakened vitality of the chicks of lot 1, as shown by their weight record and general condition. However, we do not attribute it to any toxic action of the soy bean.

(4) Baking a grain mixture composed of equal parts of soybeans, wheat, wheat bran, sunflower seed, hempseed and cracked corn, moistened with water, materially lowers its efficiency as a food, when all else entering into the diet is sufficient.

(5) The growth and development of any animal is largely dependent on its individual vitality, and for this reason it would seem desirable to use a large number in experiments on nutrition.



PLATE 42

- A.—Lot 1 at the age of three months.
- B.—Lot 3 at the age of three months.
- C.—Lot 2 at the age of three months.

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## AMMONIFICATION OF MANURE IN SOIL

By H. J. CONN, Associate Bacteriologist, and J. W. BRIGHT, Assistant Bacteriologist,  
New York State Agricultural Experiment Station

### FOREWORD

A recent series of papers of the New York Agricultural Experiment Station (8-11)<sup>1</sup> contained the results of a study of the microscopic flora of the soil. The microorganisms of the soil were classified into a few large groups, some of which were further subdivided, and in a few cases the classification was carried as far as the recognition of species. This preliminary work was considered necessary before studying the different groups with the object of recognizing more of the individual species and learning their functions.

A complete study of all soil microorganisms would be an endless task, and, indeed, rather unprofitable, provided the order of studying the different types were left entirely to chance. To begin a study of this kind, therefore, those organisms should be selected that are presumably important. It is difficult to judge, *a priori*, the importance of any particular microorganism in the soil, but a hint can be obtained by observing which types predominate in natural soil under conditions of considerable importance in practice. The organisms chosen for investigation in the present work were found to multiply in freshly manured soil. In such soil, ammonification and other forms of decomposition are vigorous and there is good reason to believe that the most rapidly multiplying organisms are of practical importance. Upon adding manure to soil several kinds of bacteria have been found to multiply strikingly, but many of them are difficult to recognize and especially difficult to describe so that others may recognize them. It has seemed unwise to make a detailed study of any organism which could not be recognized by other workers; and the work has therefore been limited for the present to two types, both of which have been identified with previously described forms.

The two bacteria investigated belong to the group of non-spore-formers (discussed in an earlier publication (10) as one of the three large groups of soil microorganisms) and more specially to that division of this group described as rapid liquefiers (p. 10, 6-9). One of them is *Pseudomonas fluorescens* (Flügge) Migula, described on page 6 of that bulletin. The second is described on page 8 of the same bulletin as the "orange liquefying type," and has now been identified with *Bacillus caudatus* Wright. As a single polar flagellum is present, it is renamed "*Pseudomonas caudatus* (Wright)."

<sup>1</sup> Reference is made by number (italic) to "Literature cited," p. 347-350.

The present paper is divided into two sections. The first shows the predominance of these two organisms in manured soil and gives the results of an investigation of their function in soil. The second gives a detailed description of the two organisms to aid in their identification by others.

H. J. CONN.

## I.—WHAT SOIL ORGANISMS TAKE PART IN THE AMMONIFICATION OF MANURE?

By J. W. BRIGHT

### INTRODUCTION

The importance of the ammonification process in the soil has long been recognized, although there has been a tendency on the part of investigators to regard it as secondary in importance to nitrification in soil fertility. Gainey (19), however, has recently claimed that the fertility of a soil is limited by processes which precede nitrification—namely, ammonification—rather than by nitrification itself.

The present work has been undertaken for the purpose of determining some of the organisms which actually cause the ammonification of manure in soil under natural conditions; to ascertain the extent to which they can carry on this ammonification; and to compare them with other organisms known to possess the power of ammonifying laboratory media.

A survey of the literature suggests that the active ammonifying organisms in the soil are generally spore formers. This idea seems to be based principally upon the conclusion reached by Marchal (36) that the spore-forming *Bacillus mycoides* is one of the most common of the soil organisms and the one that attacks protein most energetically. It should be noted, however, that he worked with a miscellaneous group of organisms and of his eight most important ammonifiers only one, the non-spore-former *B. fluorescens liquefaciens*, is a typical soil organism. J. G. Lipman (33) assumed that the spore formers were important ammonifiers, as is evidenced by the fact that he referred to the *B. subtilis* group and the streptothrices as being the most prominent ammonifying organisms numerically important in arable soils. Stephens and Withers (48) and C. B. Lipman (32) also assumed this when they decided to use *B. subtilis* as the organism with which to do their experimental work on ammonification.

That this idea is still held by some soil bacteriologists is shown by the fact that in a recent investigation by Neller (39) (an associate of J. G. Lipman), the spore-forming organisms *B. subtilis*, *B. subtilis*, *B. mycoides*, and *B. megatherium* are used to represent "some of the more common species of soil organisms" causing ammonification in the soil.

While it is undoubtedly true that a great many spore-forming organisms are capable of very active ammonification in manured soil, yet there is good reason to doubt their activity under natural conditions. Conn (6) has already pointed out that the spore formers probably exist in the soil almost entirely as spores rather than as vegetative cells and that their status as active ammonifiers in soil is doubtful. He further shows (10) that the non-spore formers not only exist in the soil in great numbers but that one group of them at least have proteolytic powers.

- \* One of this group, *Pseudomonas fluorescens*, is known to be an ammonifier. This, together with the fact that the non-spore formers have been found to be especially abundant in freshly manured soil suggests that they may be among the important soil ammonifiers. The present work was planned to test whether this assumption is correct, and, if so, to obtain as rigid proof as possible of the ammonifying agency of the non-spore-forming organisms.

#### TECHNIC

The soil used throughout the series of experiments was Dunkirk silty clay loam<sup>1</sup> obtained from a plot on the station grounds. This soil was mixed with fresh horse manure or fresh cow manure, always in the proportion of 20 parts of soil to 1 part of manure.

All samples were plated according to the usual methods, with at least two dilutions. The degree of dilution depended upon the character of the samples to be plated. Four plates were made from each dilution used and the average count of the four plates was taken to represent the count for that dilution. Whenever possible, the count was based upon the dilution averaging between 30 and 150 colonies per plate. In some cases, however, it was necessary to take into account plates which varied from these limits. In a few cases where plates were lost on account of contamination or liquefaction, the count represents an average of three instead of four plates.

The medium used in all the plating was "tap-water gelatin" made by dissolving 200 gm. of "gold-label" gelatin in 1 liter of tap water, adjusting the reaction to about  $P_H = 6.8$ , with bromthymol blue as the indicator, and clarifying with white of egg.

Nearly all of the plate counts were checked by direct microscopic examination of the soil according to the method described by Conn (12). An infusion of the soil to be examined was made by shaking up 1 gm. of the soil in 9.5 cc. of a fixative prepared by dissolving 0.15 gm. of gelatin in 1,000 cc. of hot water. Of this infusion 0.01 cc. was measured out with a capillary pipette and smeared evenly over an area of 1 square centimeter on a glass slide. This smear was then dried and stained with hot rose Bengal for 1 minute.

For all pure culture studies the manured soil was placed in small Erlenmeyer flasks, 150 gm. per flask. These were then plugged with cotton and sterilized in the autoclave at 15 pounds' pressure for two hours. Subsequent platings proved that in this way all organisms and spores were killed. The infusion for inoculating the soil was prepared as follows: A freshly streaked culture of the organism was suspended in sterile water, and the number of organisms per cubic centimeter of this

<sup>1</sup> Described according to the system of the Bureau of Soils of the U. S. Dept. of Agriculture. (MARBOT, Curtis F., BENNETT, Hugh H., LAPHAM, J. E., and LAPHAM, M. H. SOILS OF THE UNITED STATES. U. S. Dept. Agr. Bur. Soils Bul. 96, 791 p., 1913. CARR, M. Earl, LEE, Ota. jr., MAYNADIER, GUSTAVUS B., HALLOCK, D. J., and FROST, V. J. SOIL SURVEY OF ONTARIO COUNTY, NEW YORK. U. S. Dept. Agr. Bur. Soils, Adv. Sheets, Field Oper. 1910, 55 p., 1 fig., 1 map. 1912.)

infusion determined by the above microscopic method. The infusion was diluted to the desired strength and 1 cc. of it introduced into each flask. The flasks were then incubated at room temperature and studied at specified intervals. All flasks were controlled by uninoculated flasks as controls.

The method used for the determination of the ammonia produced was practically that of Potter and Snyder,<sup>1</sup> which is an adaptation of the Folin<sup>2</sup> aeration method. A number of alternating Kjeldahl flasks and absorption cylinders were set up in series so that a continuous current of air could be passed through the system. Twenty-five-gm. samples of the soils to be tested were placed in the Kjeldahl flasks and 200 cc. of *N*/50 hydrogen sulphate ( $H_2SO_4$ ) were put in each absorption cylinder. The flasks and cylinders were so connected that the air from the end flask was driven over into its adjoining cylinder and absorbed in the standard acid. Arranged in this way each Kjeldahl flask and adjacent absorption cylinder with the connecting tubes made one complete unit and any number of these units could be connected in the series.

When the apparatus was set up and all was in readiness for the aeration, 2 gm. of sodium carbonate ( $Na_2CO_3$ ), and 50 cc. of ammonia-free water were introduced into each Kjeldahl flask. The flasks were then tightly stoppered, and the air was turned on at such a rate that about 6 liters of air per minute passed through the system. The aeration was continued for about two hours and the standard acid in the absorption cylinders titrated against *N*/50 sodium hydroxid ( $NaOH$ ) to determine the amount of ammonia driven off from the soil. Care was taken to have the system absolutely air-tight and all rubber connections dry so that in passing from the flasks to the cylinders none of the ammonia would be absorbed by the water. Absorption in the standard acid was aided by using Folin ammonia tubes to break up the bubbles of air when they entered the absorption cylinders.

The determination of the amount of free ammonia in soil has always been a difficult one. The accuracy of the results obtained is somewhat doubtful, as many of the protein substances present in soil are readily broken up by the reagents used in determining the ammonia present. Consequently the ammonia determinations in this series of experiments can not be regarded as absolutely true determinations of "ammonia production." Still other factors which might tend to destroy the accuracy of the determinations are, first, that the organisms themselves might utilize the ammonia as rapidly as it is produced; and second, that it might escape into the air. The latter is improbable because the ammonia would be more likely to be absorbed by the water present in the soil. Controls of sterilized manured soil were always run at the same time as the inoculated soils, and in this way it was possible to determine whether or not the organisms in the inoculated soil affected the amount of ammonia production in any way.

<sup>1</sup> POTTER, R. S., and SNYDER, R. S. THE DETERMINATION OF AMMONIA IN SOILS. IOWA AGR. EXP. STA. RESEARCH BUL. 17, 19 p., illus. 1914.

<sup>2</sup> FOLIN, OTTO. NEUE METHODEN FÜR BESTIMMUNG DES AMMONIAKS IM HARNE UND ANDEREN FLÜSSIGKEITEN. *In* Ztschr. Physik. Chem., Bd. 37, Heft 2, p. 161-175. 1902.



## RELATIVE NUMBERS OF NON-SPORE-FORMING AND SPORE-FORMING BACTERIA IN FRESHLY MANURED SOIL

Work done by Conn (10, table 3) on the flora of freshly manured soil, previous to the present series of experiments, offers striking evidence that the non-spore-forming organisms predominate in such soil. During his work the manured soil was plated at intervals extending over a period of six months. On the third day it was found that almost 99 per cent of the entire flora was composed of non-spore-forming organisms. The present work on the flora of freshly manured soil includes experiments designed to verify these earlier results.

The method of procedure in these later experiments was practically the same throughout, except for a few differences in the treatment of samples. Soil was mixed with fresh horse manure or fresh cow manure and, with the exception of the first experiment, the manured soil was then divided into two portions, one of which was placed in an open pot and one in a flask plugged with cotton. In the first experiment the manured soil was kept only in open pots. The moisture content of the pots was controlled somewhat by frequent additions of water to replace that lost by evaporation, but no allowance was made for this in the flasks. Platings were made at frequent intervals at the first of each experiment and at longer intervals as the experiment proceeded. The experiment recorded in Table I was carried on under conditions exactly analogous to those under which Conn did his previous work, and its purpose was the verification of that work. The experiments recorded in Tables II and III were also carried on under similar conditions except that soil mixed with cow manure was used as well as that mixed with horse manure and samples were plated from plugged flasks as well as from open pots.

TABLE I.—Plate counts of the microorganisms in manured soil in open pots. Experiment I

[Counts indicate numbers of colonies per gram of soil]

Time since adding manure to soil.	Total count.	Actinomycetes.		Non-spore formers.		Spore formers.	
		Plate count.	Percentage of total flora.	Plate count.	Percentage of total flora.	Plate count.	Percentage of total flora.
<i>Days.</i>							
2.....	60,000,000	4,000,000	7.5	56,000,000	92.5	None.	0.0
3.....	50,000,000	6,000,000	7.5	74,000,000	92.5	None.	.0
4.....	125,000,000	5,000,000	4.0	116,000,000	92.8	4,000,000	3.3
6.....	215,000,000	6,000,000	2.6	220,500,000	91.5	9,500,000	3.9
9.....	45,000,000	5,000,000	11.1	38,500,000	85.5	1,500,000	3.4
13.....	41,000,000	4,000,000	9.3	36,500,000	85.0	1,500,000	5.7
16.....	35,000,000	12,000,000	34.3	23,000,000	65.7	None.	.0
21.....	50,000,000	13,000,000	26.0	35,000,000	70.0	2,000,000	4.0
24.....	55,000,000	11,500,000	20.6	43,500,000	72.4	None.	.0
29.....	85,000,000	8,500,000	10.0	76,500,000	90.0	None.	.0
35.....	45,000,000	13,000,000	29.0	32,000,000	71.0	None.	.0
58.....	95,000,000	8,500,000	8.9	83,500,000	88.0	3,000,000	3.1
94.....	18,000,000	5,500,000	30.5	11,500,000	63.8	1,000,000	5.7
113.....	20,000,000	5,000,000	25.0	15,000,000	75.0	None.	.0
Average.....			16.3		81.6		2.1

TABLE II.—Plate counts of the microorganisms in manure soil in open pots and closed flasks. Experiment II

## COW MANURE

(Counts indicate number of colonies per gram of soil)

Treatment and time since adding manure to soil.	Total count.	Actinomycetes		Non-spore formers		Spore formers	
		Plate count.	Per-centage of total flora	Plate count	Per-centage of total flora	Plate count	Per-centage of total flora
Open pots:							
1 day.....	164,000,000	9,500,000	5.6	144,000,000	94.4	None	0.0
2 days.....	91,000,000	10,500,000	11.5	80,500,000	88.5	Trace	.4
3 days.....	96,500,000	10,000,000	11.0	86,500,000	89.0	2,000,000	2.0
4 days.....	127,000,000	8,000,000	7.0	117,000,000	91.8	1,000,000	.8
5 days.....	55,000,000	7,500,000	14.4	47,500,000	86.8	1,000,000	1.8
7 days.....	490,000,000	35,000,000	6.8	455,000,000	92.0	1,000,000	.2
8 days.....	251,000,000	40,000,000	15.0	211,000,000	83.0	3,000,000	1.2
9 days.....	52,000,000	7,000,000	12.4	45,000,000	86.2	1,000,000	.2
11 days.....	412,000,000	100,000,000	24.1	312,000,000	75.1	2,000,000	.5
13 days.....	67,000,000	16,000,000	23.1	51,000,000	75.2	500,000	.7
15 days.....	395,000,000	160,000,000	40.0	235,000,000	58.7	3,000,000	.7
17 days.....	245,000,000	50,000,000	22.6	195,000,000	78.0	3,000,000	1.4
Average.....			18.2		80.9		.9
Closed flasks:							
1 day.....	27,500,000	5,750,000	18.5	21,750,000	81.7	None	0.0
2 days.....	64,000,000	6,000,000	10.0	57,000,000	90.0	None	0.0
3 days.....	67,000,000	6,000,000	9.0	59,000,000	88.1	1,000,000	1.5
4 days.....	81,000,000	15,000,000	18.6	66,000,000	79.0	2,000,000	2.4
5 days.....	101,000,000	15,000,000	14.0	86,000,000	84.1	1,000,000	1.0
7 days.....	42,000,000	12,500,000	28.5	29,500,000	69.2	1,000,000	2.3
8 days.....	65,500,000	12,000,000	18.1	53,500,000	81.0	Trace	.2
9 days.....	34,500,000	9,000,000	24.1	25,500,000	72.5	1,000,000	2.8
11 days.....	330,000,000	260,000,000	78.3	70,000,000	21.1	2,000,000	.6
13 days.....	330,000,000	300,000,000	90.9	30,000,000	9.0	1,000,000	.3
15 days.....	330,000,000	225,000,000	68.8	105,000,000	31.0	0,000,000	0.0
17 days.....	350,000,000	100,000,000	28.6	250,000,000	71.4	2,000,000	.6
Average.....			24.3		66.1		1.5

## HORSE MANURE

<b>Open pots:</b>							
1 day.....	300,000,000	9,500,000	3.2	290,500,000	96.8	None	0.0
2 days.....	100,000,000	18,000,000	18.0	82,000,000	82.0	2,000,000	2.0
3 days.....	157,000,000	12,000,000	7.4	145,000,000	91.7	1,000,000	.6
4 days.....	907,000,000	22,500,000	2.3	884,500,000	97.0	2,000,000	.2
5 days.....	775,000,000	16,000,000	2.1	759,000,000	91.6	2,000,000	.2
7 days.....	615,000,000	65,000,000	10.4	550,000,000	88.0	4,000,000	.6
8 days.....	67,500,000	6,000,000	8.9	61,500,000	90.4	1,000,000	.7
9 days.....	460,000,000	81,000,000	17.7	379,000,000	81.2	3,000,000	.8
11 days.....	740,000,000	115,000,000	15.0	625,000,000	82.0	3,000,000	.4
13 days.....	376,000,000	100,000,000	26.0	276,000,000	71.6	2,000,000	.5
15 days.....	255,000,000	94,000,000	37.0	161,000,000	62.2	2,000,000	.8
17 days.....	1,705,000,000	700,000,000	41.0	1,005,000,000	58.2	1,000,000	.1
Average.....			16.1		81.1		.6
<b>Closed flasks:</b>							
1 day.....	63,000,000	11,000,000	17.5	52,000,000	82.5	None	0.0
2 days.....	38,000,000	7,000,000	18.0	31,000,000	81.0	Trace	.1
3 days.....	82,000,000	8,000,000	9.0	74,000,000	89.2	2,000,000	.2
4 days.....	78,000,000	13,000,000	16.0	65,000,000	84.0	2,000,000	.2
5 days.....	316,000,000	100,000,000	31.0	216,000,000	67.0	2,000,000	.6
7 days.....	810,000,000	185,000,000	22.0	625,000,000	76.0	2,000,000	.3
8 days.....	75,500,000	24,000,000	31.4	51,500,000	67.0	1,000,000	1.3
9 days.....	58,500,000	20,000,000	33.0	38,500,000	64.0	2,000,000	.3
11 days.....	375,000,000	210,000,000	55.0	165,000,000	43.0	2,000,000	.5
13 days.....	160,000,000	85,000,000	52.0	75,000,000	45.0	2,000,000	.4
15 days.....	1,180,000,000	800,000,000	67.0	380,000,000	31.0	1,000,000	.1
17 days.....	1,045,000,000	800,000,000	75.0	245,000,000	23.0	1,000,000	.1
Average.....			31.8		65.4		.6

TABLE III.—Plate counts of the microorganisms in manured soil in open pots and closed flasks. Experiment III

## COW MANURE

[Counts indicate number of colonies per gram of soil]

Treatment and time since adding manure to soil.	Total count.	Actinomycetes.		Non-spore formers.		Spore formers.	
		Plate count.	Per-centage of total flora.	Plate count.	Per-centage of total flora.	Plate count.	Per-centage of total flora.
Open pots:							
1 day	58,000,000	8,000,000	13.8	48,000,000	82.7	2,000,000	3.5
2 days	72,150,000	5,000,000	6.7	68,000,000	95.1	Trace.	.3
3 days	497,500,000	15,000,000	3.0	482,500,000	97.0	None.	.0
4 days	91,000,000	12,000,000	13.2	81,000,000	87.1	None.	.0
5 days	102,500,000	12,500,000	12.2	90,000,000	87.8	None.	.0
7 days	15,250,000	5,000,000	33.5	11,250,000	86.5	None.	.0
8 days	43,000,000	5,000,000	11.7	38,000,000	88.3	None.	.0
9 days	17,500,000	2,500,000	14.3	15,000,000	85.7	None.	.0
10 days	40,000,000	12,000,000	30.0	28,000,000	70.0	None.	.0
12 days	90,000,000	15,000,000	15.7	81,000,000	84.3	None.	.0
15 days	187,000,000	20,000,000	10.7	167,000,000	89.3	None.	.0
18 days	75,000,000	20,000,000	26.7	55,000,000	73.3	None.	.0
21 days	64,000,000	10,000,000	14.4	52,000,000	81.3	1,500,000	2.3
Average			14.3		85.3		.4
Closed flasks:							
1 day	51,000,000	8,000,000	15.7	41,000,000	81.4	1,500,000	2.9
2 days	43,000,000	6,000,000	14.3	35,000,000	84.5	500,000	1.2
3 days	295,000,000	10,000,000	3.4	285,000,000	96.6	None.	.0
4 days	68,000,000	30,000,000	44.1	38,000,000	55.9	None.	.0
5 days	44,000,000	11,000,000	25.5	33,000,000	74.5	None.	.0
7 days	27,000,000	10,000,000	37.0	17,000,000	62.4	None.	.0
8 days	45,500,000	23,000,000	50.5	22,500,000	49.4	None.	.0
9 days	31,000,000	20,000,000	64.6	11,000,000	35.4	None.	.0
10 days	36,500,000	17,500,000	48.0	19,000,000	52.0	None.	.0
12 days	29,500,000	20,000,000	68.0	9,500,000	32.2	None.	.0
15 days	13,000,000	25,000,000	47.2	28,000,000	51.8	None.	.0
18 days	125,000,000	65,000,000	52.0	60,000,000	48.0	None.	.0
21 days	46,000,000	25,000,000	54.4	21,000,000	45.6	None.	.0
Average			38.3		61.4		.3

## HORSE MANURE

<b>Open pots:</b>							
1 day	110,000,000	12,000,000	10.9	97,000,000	88.1	750,000	1.0
2 days	126,000,000	7,500,000	6.3	118,500,000	93.5	1,500,000	1.2
3 days	195,000,000	10,000,000	5.0	185,000,000	95.0	None.	.0
4 days	150,000,000	15,000,000	10.0	135,000,000	90.0	None.	.0
5 days	123,000,000	12,000,000	9.9	110,000,000	90.1	None.	.0
7 days	95,000,000	15,000,000	15.8	80,000,000	84.2	None.	.0
8 days	300,000,000	35,000,000	11.7	265,000,000	88.3	None.	.0
9 days	30,500,000	5,000,000	16.4	25,500,000	83.6	None.	.0
10 days	59,000,000	5,000,000	8.5	54,000,000	91.5	None.	.0
12 days	48,000,000	20,000,000	41.7	28,000,000	58.3	None.	.0
15 days	243,500,000	40,500,000	16.6	203,000,000	83.9	None.	.0
18 days	130,000,000	85,000,000	65.0	45,000,000	34.5	None.	.0
21 days	117,500,000	30,000,000	25.5	87,500,000	74.5	500,000	.5
Average			14.9		84.9		.2
<b>Closed flasks:</b>							
1 day	40,000,000	4,500,000	11.3	34,500,000	86.3	1,000,000	2.5
2 days	185,500,000	8,000,000	4.3	177,500,000	94.0	1,000,000	1.7
3 days	82,500,000	10,000,000	12.1	72,500,000	87.9	None.	.0
4 days	75,000,000	22,500,000	30.0	52,500,000	70.0	None.	.0
5 days	33,000,000	13,000,000	39.4	20,000,000	60.6	None.	.0
7 days	515,000,000	375,000,000	72.9	140,000,000	27.1	None.	.0
8 days	605,000,000	150,000,000	24.8	455,000,000	75.2	None.	.0
9 days	62,500,000	32,000,000	51.2	30,500,000	48.8	None.	.0
10 days	780,000,000	600,000,000	76.9	180,000,000	23.1	None.	.0
12 days	87,000,000	50,000,000	57.4	37,000,000	42.6	None.	.0
15 days	41,500,000	27,500,000	66.3	14,000,000	33.8	None.	.0
18 days	100,000,000	65,000,000	65.0	35,000,000	35.0	None.	.0
21 days	40,000,000	30,000,000	75.0	10,000,000	25.0	None.	.0
Average			46.2		53.5		.3

A survey of the results in Tables I, II, and III, shows that the number of non-spore formers in the open pots of manured soil increased rapidly for the first few days (see Table I, column 5). In every instance the highest percentage of this group of organisms was reached within the first week after the addition of the manure and this maximum point was never less than 92.5 per cent, while in some cases it reached 97 per cent. The results in the flasks were much more erratic and, while the percentage of non-spore formers often ran above 90 per cent of the flora, the lines of increase and decrease were not so well marked as they were in the pot experiments. This was undoubtedly due to the fact that conditions of aeration and moisture content were decidedly abnormal. By summarizing the three tables it was found that the non-spore-forming organisms averaged 74.1 per cent of the entire flora in both the pots and flasks; the Actinomycetes 25.1 per cent, and the spore formers only 0.8 per cent.

TABLE IV.—Results of the isolation of organisms from manured soil

Source			Open pots				Closed flasks			
Sample No.	Kind of manure	Time since adding manure	Total count	Number of organisms isolated	Number which grew on agar <sup>a</sup>	Number of non-spore formers	Total count	Number of organisms isolated	Number which grew on agar <sup>a</sup>	Number of non-spore formers
1	Horse	6	251,000,000	14	12	10	117,000,000			
2	do.	10	89,000,000	20	20	20	95,000,000			
3	do.	27	71,000,000	25	24	21	49,000,000	17	17	17
4	do.	22	37,000,000	20	20	19	28,000,000	5	5	5
5	do.	27	30,000,000	27	27	27	15,000,000			
6	Cow	9	231,000,000	37	36	35				
7	do.	11					137,000,000	41	41	39
8	do.	23	174,000,000	15	14	13	141,500,000	15	14	14
9	do.	3	68,500,000	15	14	14	67,000,000	14	14	14
10	Horse	8	67,000,000	7	6	6	74,500,000	9	9	9
11	Cow	8	251,000,000	8	8	8	61,500,000	4	4	4
12	Horse	24	1,705,000,000	13	11	11	1,341,000,000	11	11	11
13	Cow	24	245,000,000	11	10	10	110,000,000	9	9	9
14	Horse	8	300,000,000	3	3	3	664,000,000	8	8	8
15	Cow	8	41,000,000	10	10	10	41,500,000			
				265	254	247		170	160	157

<sup>a</sup> Plain nutrient agar was used as medium for isolated colonies.

While the data accumulated in the preceding experiments indicated very strongly that the non-spore formers were the predominating organisms in the manured soil, yet the proof was not absolute, because it was based entirely upon the appearance of the colonies upon the plates. Those colonies which possessed the characteristic spreading or filamentous appearance of the typical spore formers were classified accordingly; but some non-spore formers may thus have been inadvertently recorded as spore formers, or some spore formers as non-spore formers. A number of isolations were made, therefore, from the plates poured during the series of experiments described above. All colonies which looked like

spore formers were transferred to agar slants, as were a representative number of colonies of other types. About 97 per cent of these cultures grew and were subsequently examined under the microscope for spore formation.

Table IV, which contains the recorded data from this experiment, shows that of the 254 organisms from the open pots which grew after isolation, only 2.8 per cent were spore formers, and of the 160 organisms from the flasks which grew after isolation only 1.8 per cent were spore formers. And this despite the fact that a special effort was made to include all those colonies whose appearance suggested that they might be spore-forming organisms.

#### GROWTH OF *PSEUDOMONAS FLUORESCENS* AND *PS. CAUDATUS* COMPARED WITH THE GROWTH OF *BACILLUS CEREUS* IN STERILIZED MANURED SOILS

The organisms selected for the rest of the work were two non-spore formers, *Pseudomonas fluorescens* (Flügge) Migula and *Ps. caudatus* (Wright) Conn, and a spore former, *Bacillus cereus* Frankland.<sup>1</sup> The first two organisms are described in the second section of this article, and were chosen because of the frequency of their occurrence in manured soil. *B. cereus*, according to Conn (9) and Laubach and Rice (27), is a typical spore former occurring in soil and was selected for the purpose of comparison with these organisms.

#### SOIL INOCULATED WITH THE THREE ORGANISMS SEPARATELY

In the series of experiments designed to show the relative rates of growth of the three organisms under investigation, manured soil was sterilized in flasks and inoculated with pure cultures in suspensions of carefully determined strength. Samples from each series were plated at similar intervals, and an effort was made to make all results comparable. Microscopic counts were made of all the samples of soil inoculated with *B. cereus* in order to determine the number of vegetative cells actually present in the soil. As *Ps. fluorescens* and *Ps. caudatus*, on the other hand, grow well on plates, form no spores, and show no tendency to clump, a microscopic count of them was not so important as the plate count, and since they are so small as to be easily overlooked under the microscope a microscopic count proved even less accurate than the plate count. The results as set forth in Table VI, Experiment I, show that *Ps. caudatus* increased from a 13,300,000 plate count on the day of inoculation to a 1,720,000,000 count seven days later, or an increase of 132 times the original inoculation. The initial plate count of *Ps. fluorescens* was 4,390,000, and on the seventh day the count was 475,000,000, an increase of 110 times the original count. *B. cereus*, on the

<sup>1</sup> As identified by Conn. This organism agrees with the descriptions of Chester (2, p. 278), and Laurence and Ford (28, p. 284-287).

other hand, showed a much lower rate of increase and developed from an initial plate count of 1,800,000 (see Table V, Experiment I) to a count of 15,000,000 on the seventh day, an increase of 8.3 times the original inoculation. The microscopic count on the seventh day showed 36,000,000 vegetative cells, an increase of 20 times the initial count. In the four series *Ps. caudatus* showed its greatest increase in Experiment III (Table IV), on the ninth day, when it showed a count 913 times greater than its initial count; *Ps. fluorescens* registered its greatest increase in Experiment IV on the fourteenth day, when it showed a count 530 times higher than the original count; and *B. cereus* made its greatest increase in Experiment IV (Table V) on the sixteenth day, showing a count 29 times higher than the original.

TABLE V.—Multiplication of *B. cereus* inoculated into sterile manured soil

[Count indicates the numbers per gram of soil]

Experiment No. and time since inoculation.	Plate count.	Microscopic count			
		Groups		Individuals	
		Vegetative cells	Spores	Vegetative cells	Spores
Experiment I:				# 1,800,000	None
0 days.....	3,000,000	5,000,000	19,000,000	9,000,000	23,000,000
3 days.....	1,750,000	6,000,000	6,000,000	3,000,000	6,000,000
5 days.....	15,000,000	30,000,000	17,000,000	50,000,000	18,000,000
7 days.....					
Experiment II: <sup>b</sup>				# 1,300,000	None
0 days.....	2,800,000				
5 days.....	4,000,000				
8 days.....	7,000,000				
17 days.....	12,000,000				
23 days.....					
Experiment III:				# 115,000	None
0 days.....	4,000,000	7,000,000	18,000,000	11,000,000	33,000,000
2 days.....	5,000,000	3,100,000	11,100,000	10,500,000	10,100,000
4 days.....	10,500,000	2,100,000	16,000,000	11,500,000	19,900,000
6 days.....	10,000,000	2,500,000	14,500,000	7,500,000	18,000,000
8 days.....					
Experiment IV:				# 101,000	None
0 days.....	8,000,000	2,000,000	5,000,000	11,500,000	30,000,000
2 days.....	5,000,000	1,100,000	13,000,000	13,700,000	24,100,000
4 days.....	12,500,000	2,500,000	11,000,000	9,100,000	27,500,000
6 days.....	17,000,000	1,100,000	9,000,000	4,400,000	27,000,000
8 days.....	17,000,000	2,000,000	15,000,000	6,000,000	19,000,000
11 days.....	17,000,000	2,500,000	12,000,000	3,000,000	21,000,000
13 days.....	21,000,000	2,100,000	15,500,000	6,500,000	26,500,000
14 days.....	20,000,000	3,000,000	21,000,000	6,500,000	27,000,000
16 days.....					

<sup>a</sup> Computed from the number of organisms in the infusion used for inoculation.  
<sup>b</sup> No microscopic count made.

## SOIL INOCULATED WITH A MIXTURE OF THE THREE ORGANISMS

Table VII gives the results of placing the three organisms in competition one with another by inoculating flasks of sterile manured soil with all of them together. Infusions were made from fresh cultures of each organism and the strength of these infusions determined by the microscopic method. After infusions of the proper strength had been obtained, equal amounts of each were thoroughly mixed and 1 cc. of the mixture

added to the flasks containing 150 gms. of sterile manured soil. These flasks were then incubated at room temperature and plates and smears made from them at regular intervals. In Experiment I, Table VII, the ratio between the numbers of organisms of *Ps. fluorescens*, *Ps. caudatus*, and *B. cereus* was 1 to 1 to 1; in Experiment II the ratio was 1 to 8 to 33; in Experiment III the ratio was 1 to 7 to 33. Although *B. cereus* was as abundant as the other organisms in Experiment I and was much more numerous than they in the later experiments, it failed to appear upon any of the plates poured. The non-spore-forming organisms multiplied very rapidly, and in Experiment II, *Ps. fluorescens* developed from an initial count of 30,000 to a maximum count of 560,000,000 on the third day, an increase of over 18,500 times its count at the time of inoculation. In the same experiment *Ps. caudatus* developed from an initial count of 180,000 to a maximum count of 1,190,000,000 on the seventh day, an increase of 6,600 times its count at the time of inoculation. The microscopic examination of the smears made during this series of experiments showed that the vegetative cells of *B. cereus* rapidly decreased in numbers and in a few days the organism could be identified only in the spore form, while the non-spore formers, especially *Ps. caudatus*, showed a steady increase in numbers for several days.

TABLE VI.—Multiplication of non-spore formers inoculated into sterile manured soil

[Count indicates numbers per gram of soil]

Experiment No. and time since inoculation.	<i>Pseudomonas fluorescens</i> .			<i>Pseudomonas caudatus</i> .		
	Plate count.	Microscopic count.		Plate count.	Microscopic count.	
		Groups.	Individuals.		Groups.	Individuals.
Experiment I:						
0 days.....			<sup>a</sup> 4,390,000			<sup>a</sup> 13,300,000
3 days.....	260,000,000	187,000,000	204,000,000	665,000,000	492,000,000	608,000,000
5 days.....	185,000,000	133,000,000	152,000,000	4,800,000,000	1,340,000,000	1,614,000,000
7 days.....	475,000,000	259,000,000	335,000,000	1,730,000,000	1,105,000,000	1,354,000,000
Experiment II: <sup>b</sup>						
0 days.....			<sup>a</sup> 3,300,000			<sup>a</sup> 6,600,000
5 days.....	145,000,000			2,700,000,000		
8 days.....	160,000,000			1,500,000,000		
13 days.....	200,000,000			No count.		
17 days.....	210,000,000			4,000,000,000		
23 days.....	300,000,000			No count.		
Experiment III:						
0 days.....			<sup>a</sup> 1,600,000			<sup>a</sup> 1,600,000
2 days.....	140,000,000	79,000,000	88,000,000	1,440,000,000	718,000,000	728,000,000
4 days.....	260,000,000	173,000,000	188,000,000	1,340,000,000	760,000,000	784,000,000
9 days.....	315,000,000	210,000,000	247,000,000	1,460,000,000	736,000,000	808,000,000
11 days.....	350,000,000	310,000,000	348,000,000	1,190,000,000	794,000,000	822,000,000
Experiment IV: <sup>b</sup>						
0 days.....			<sup>a</sup> 1,000,000			<sup>a</sup> 1,050,000
2 days.....	101,000,000			114,000,000		
4 days.....	390,000,000			181,000,000		
6 days.....	395,000,000			194,000,000		
8 days.....	470,000,000			220,500,000		
10 days.....	375,000,000			205,000,000		
12 days.....	400,000,000			270,000,000		
14 days.....	510,000,000			269,000,000		
15 days.....	470,000,000			171,500,000		

<sup>a</sup> Computed from the number of organisms in the infusion used for inoculation.  
<sup>b</sup> No microscopic count made.

TABLE VII.—Plate counts of the microorganisms in sterile manured soil inoculated with a mixture of one spore former and two non-spore formers

[Count indicates number of colonies per gram of soil]

Time since inoculation.	Experiment I			Experiment II			Experiment III		
	<i>Pseudo-monas caudatus</i>	<i>Pseudo-monas fluorescens</i>	<i>Bacillus cereus</i>	<i>Pseudo-monas caudatus</i>	<i>Pseudo-monas fluorescens</i>	<i>Bacillus cereus</i>	<i>Pseudo-monas caudatus</i>	<i>Pseudo-monas fluorescens</i>	<i>Bacillus cereus</i>
Days.									
0	0 250,000	0 100,000		0 150,000	0 10,000	0 50,000	0 1,500,000	0 250,000	0 500,000
1	None.	194,000,000		10,000,000	21,000,000		None.	1,000,000	1,000,000
2	None.	188,000,000		89,000,000	100,000,000		No test	No test	No test
3	None.	960,000,000		997,000,000	570,000,000		14,000,000	0,000,000	0,000,000
4	80,000,000	240,000,000		400,000,000	150,000,000		199,000,000	15,000,000	15,000,000
5	480,000,000	120,000,000		575,000,000	150,000,000		No test	No test	No test
6	590,000,000	220,000,000		470,000,000	180,000,000		No test	No test	No test
7	570,000,000	115,000,000		1,100,000,000	450,000,000		No test	No test	No test
8	710,000,000	100,000,000		1,095,000,000	500,000,000		No test	No test	No test
9	770,000,000	110,000,000		1,010,000,000	110,000,000		No test	No test	No test
10	865,000,000	72,500,000		940,000,000	90,000,000		No test	No test	No test
11	920,000,000	75,000,000		900,000,000	80,000,000		120,000,000	6,000,000	6,000,000
12	110,000,000	50,000,000		680,000,000	90,000,000		100,000,000	80,000,000	80,000,000
13	880,000,000	80,000,000		910,000,000	120,000,000		100,000,000	10,000,000	10,000,000
14	810,000,000	110,000,000		730,000,000	80,000,000		100,000,000	60,000,000	60,000,000
15	870,000,000	90,000,000		660,000,000	80,000,000		No test	No test	No test
16	820,000,000	80,000,000		750,000,000	80,000,000		No test	No test	No test
17	805,000,000	80,000,000		180,000,000	30,000,000		No test	No test	No test
18	960,000,000	70,000,000		600,000,000	90,000,000		100,000,000	50,000,000	50,000,000
19	730,000,000	80,000,000		590,000,000	110,000,000		200,000,000	10,000,000	10,000,000
20	800,000,000	65,000,000		664,000,000	90,000,000		150,000,000	None	None
21				360,000,000	150,000,000		270,000,000	10,000,000	10,000,000
22				425,000,000	150,000,000		110,000,000	60,000,000	60,000,000
23				590,000,000	140,000,000		No test	No test	No test
24				715,000,000	100,000,000		100,000,000	70,000,000	70,000,000
25				710,000,000	100,000,000		No test	No test	No test
26				No test	No test		310,000,000	75,000,000	75,000,000
27				490,000,000	190,000,000		No test	No test	No test
28				510,000,000	80,000,000		490,000,000	60,000,000	60,000,000
29				430,000,000	110,000,000		No test	No test	No test
30				310,000,000	90,000,000		180,000,000	20,000,000	20,000,000
31				290,000,000	60,000,000		170,000,000	60,000,000	60,000,000
32							100,000,000	55,000,000	55,000,000

a Computed from the number of organisms in the infusion used for inoculation

The results as recorded in this series of experiments indicate quite clearly that the non-spore-forming organisms, *Ps. fluorescens* and *Ps. caudatus* rapidly gain the ascendancy over *B. cereus* when placed in competition with it in sterile freshly manured soil. The vegetative cells of *B. cereus* apparently soon sporulate and remain in the resting condition.

#### RELATIVE NUMBERS OF THE ORGANISMS IN QUESTION IN A SOIL BEFORE AND AFTER ADDING MANURE

Tables VIII, IX, X, and XI record data which show the relative numbers of *Ps. fluorescens*, *Ps. caudatus*, and *B. cereus* in soil in which no ammonification is occurring, and in the same soil after manure has been added and decomposition is occurring rapidly. Table VIII<sup>1</sup> contains the data obtained as the result of analyses of an untreated field

<sup>1</sup> The data given in this table were obtained by Com in his earlier work. Much of it has already been used in his soil-flora studies (3-11).



TABLE VIII.—Comparison between numbers of *Bacillus cereus* and the numbers of certain non-spore formers in Plot I, soil untreated

[Count indicates the number of colonies per gram of soil]

Date.	Total count.	<i>Bacillus cereus</i> .	<i>Pseudomonas fluorescens</i> .	<i>Pseudomonas caudatus</i> .
1912.				
Sept. 23.....	38,250,000	350,000	a None.	60,000
Oct. 25.....	17,000,000	150,000	150,000	None.
Dec. 3.....	35,000,000	200,000	None.	None.
17.....	23,500,000	No count.	No count.	No count.
1913.				
Jan. 15.....	17,500,000	200,000	100,000	None.
Feb. 5.....	27,500,000	350,000	None.	None.
14.....	54,000,000	300,000	300,000	None.
Mar. 11.....	29,200,000	400,000	None.	None.
Apr. 4.....	27,000,000	400,000	200,000	None.
July 10.....	22,600,000	500,000	None.	None.
Nov. 26.....	15,000,000	350,000	None.	None.
Dec. 15.....	12,400,000	200,000	Trace.	None.
1914.				
Jan. 16.....	16,150,000	700,000	None.	None.
30.....	29,300,000	500,000	Trace.	None.
Feb. 7.....	26,700,000	400,000	None.	None.
26.....	38,500,000	600,000	Trace.	None.
Apr. 15.....	19,400,000	350,000	None.	None.
29.....	16,100,000	450,000	None.	None.
Aug. 7.....	(b)	200,000	None.	None.
19.....	23,400,000	175,000	600,000	None.
1917.				
May 4.....	12,500,000	300,000	a 250,000	None.

a Dilution so great that no colonies appeared on the plates. b Count lost on account of liquefaction.

TABLE IX.—Comparison between numbers of *Bacillus cereus* and numbers of certain non-spore formers in soil from Plot I. Soil manured and kept in the laboratory. Series I

[Counts indicate number of colonies per gram of soil]

Experiment No. and time since adding manure to soil.	Pot. <sup>a</sup>					Flask. <sup>a</sup>				
	Total count.	<i>Ps. fluorescens</i> .		<i>Ps. caudatus</i> .		Total count.	<i>Ps. fluorescens</i> .		<i>Ps. caudatus</i> .	
		Plate count.	Per cent-ages of total flora.	Plate count.	Per cent-ages of total flora.		Plate count.	Per cent-ages of total flora.	Plate count.	Per cent-ages of total flora.
Experiment I:										
7 days...	351,000,000	10,000,000	4.0	None.	0.0	317,000,000	None.	0.0	9,000,000	2.8
16 days...	105,000,000	6,000,000	5.7	47,000,000	44.8	99,000,000	10,000,000	9.9	50,000,000	50.5
Experiment II:										
3 days...	89,000,000	5,000,000	5.7	25,000,000	28.2	121,000,000	10,000,000	7.0	35,000,000	28.9
13 days...	871,000,000	7,000,000	4.0	90,000,000	38.1	485,000,000	75,000,000	15.4	35,000,000	7.2
30 days...	71,000,000	3,000,000	4.2	10,000,000	13.7	43,000,000	2,000,000	4.7	4,000,000	9.5
Experiment III:										
3 days...	311,000,000	19,000,000	6.3	50,000,000	21.4	145,000,000	10,000,000	6.9	20,000,000	13.8
15 days...	11,000,000	2,000,000	5.4	5,000,000	13.5	78,000,000	4,000,000	5.1	3,000,000	3.9
30 days...	430,000,000	2,000,000	6.6	1,000,000	3.4	22,000,000	1,000,000	4.5	5,000,000	2.2
Experiment IV:										
4 days...	331,000,000	4,000,000	1.7	18,000,000	7.8	136,000,000	17,000,000	12.5	35,000,000	25.7
23 days...	174,000,000	No count.	.....	No count.	.....	143,500,000	No count.	.....	No count.	.....
Average of all experiments...			4.9		17.9			7.3		15.6

<sup>a</sup> *Bacillus cereus* did not develop on the plates.

soil from Plot I made at intervals for a period of three years. It will be noted that during that time the non-spore-forming *Ps. caudatus* appeared only once and then in comparatively small numbers; *Ps. fluorescens* appeared nine times, and only twice constituted more than 1 per cent and never more than 2.5 per cent of the total flora. It is also a significant fact that the spore-forming *B. cereus* was always present and made up from 0.55 to 4.4 per cent of the total flora. Another plating made just previous to the present work showed that the organisms were present as follows: *Ps. fluorescens*, 2 per cent; *Ps. caudatus*, less than 0.1 per cent; and *B. cereus*, 2.4 per cent.

TABLE X.—Comparison between numbers of *Bacillus cereus* and numbers of certain non-spore formers in soil from Plot I. Soil manured and kept in the laboratory. Series II

## HORSE MANURE

[Counts indicate the number of colonies per gram of soil]

Time since adding manure to soil.	Open pot <sup>a</sup>				Closed flask <sup>a</sup>			
	<i>Ps. fluorescens</i>		<i>Ps. caudatus</i>		<i>Ps. fluorescens</i>		<i>Ps. caudatus</i>	
	Total count.	Plate count.	Percentage of total flora.	Percentage of total flora.	Total count.	Plate count.	Percentage of total flora.	Percentage of total flora.
Days.								
1.....	300,000,000	Trace	0.1	Trace	0.1	61,000,000	None	0.0
2.....	109,000,000	3,000,000	1.8	None	0.4	83,000,000	1,000,000	1.2
3.....	157,000,000	7,500,000	4.7	None	0.5	83,000,000	1,000,000	1.2
4.....	907,500,000	30,000,000	3.3	Trace	0.6	78,000,000	2,000,000	2.6
5.....	775,000,000	25,000,000	3.2	Trace	1.1	116,000,000	5,000,000	4.3
7.....	625,000,000	20,000,000	3.2	20,000,000	3.2	810,000,000	2,500,000	0.3
8.....	67,500,000	750,000	1.1	1,140,000	3.6	75,500,000	None	0.0
9.....	480,000,000	12,000,000	2.5	15,000,000	3.1	48,500,000	None	0.0
11.....	740,000,000	65,000,000	8.4	8,000,000	1.1	375,000,000	750,000	0.2
13.....	176,000,000	22,500,000	6.0	7,500,000	3.0	160,000,000	1,000,000	0.6
15.....	295,000,000	5,000,000	1.7	4,000,000	1.3	1,180,000,000	2,500,000	0.2
17.....	1,705,000,000	20,000,000	1.1	50,000,000	2.9	1,045,000,000	1,000,000	0.1
Average.....			3.1		1.3			1.3

## COW MANURE

1.....	164,000,000	Trace	0.1	Trace	4.1	37,000,000	Trace	0.1	None	0.0
2.....	93,000,000	Trace	0.2	Trace	0.9	64,000,000	1,000,000	1.5	Trace	0.1
3.....	98,500,000	500,000	0.5	None	0.0	67,000,000	1,400,000	2.1	1,000,000	1.5
4.....	127,000,000	3,500,000	2.7	None	0.0	81,000,000	None	0.0	None	0.0
5.....	55,000,000	100,000	0.2	None	0.0	108,000,000	None	0.0	None	0.0
7.....	400,000,000	1,000,000	0.2	None	0.0	42,000,000	1,500,000	3.6	None	0.0
8.....	251,000,000	1,500,000	0.6	None	0.0	54,500,000	Trace	0.1	None	0.0
9.....	51,000,000	2,000,000	3.9	500,000	0.9	34,500,000	Trace	0.1	None	0.0
11.....	412,000,000	2,100,000	0.5	7,500,000	1.8	310,000,000	Trace	0.1	None	0.0
13.....	67,000,000	Trace	0.2	None	0.0	323,000,000	Trace	0.1	1,000,000	0.3
15.....	395,000,000	5,000,000	1.3	70,000,000	2.5	310,000,000	2,000,000	0.6	None	0.0
17.....	245,000,000	None	0.0	2,000,000	0.8					
Average.....			0.8		0.5					

<sup>a</sup> *B. cereus* did not develop on the plates

Tables IX, X, and XI record the results of platings made from samples of the same soil after being treated with fresh manure. Examination

of these tables show that either *Ps. fluorescens* or *Ps. caudatus*, or both, almost invariably appeared on every sample plated and often constituted as high as 15 or 20 per cent of the entire flora, while *B. cereus* was very seldom observed; in fact, very few spore formers of any type were recognized. It must be borne in mind that these data were obtained from soil in which it was definitely determined that decomposition processes were occurring.

TABLE XI.—Comparison between numbers of *Bacillus cereus* and numbers of certain non-spore formers in soil from Plot I. Soil manured and kept in the laboratory. Series III

## HORSE MANURE

[Counts indicate number of colonies per gram of soil]

Time since adding manure to soil.	Open pot. <sup>a</sup>					Closed flask. <sup>a</sup>				
	<i>Ps. fluorescens</i> .			<i>Ps. caudatus</i> .		<i>Ps. fluorescens</i> .			<i>Ps. caudatus</i> .	
	Total count.	Plate count.	Percentage of total flora.	Plate count.	Percentage of total flora.	Total count.	Plate count.	Percentage of total flora.	Plate count.	Percentage of total flora.
Days.										
1.....	110,000,000	2,000,000	1.8	1,500,000	1.4	40,000,000	750,000	1.8	1,000,000	2.5
2.....	110,000,000	6,000,000	5.0	5,000,000	4.2	182,500,000	10,000,000	5.5	25,000,000	13.7
3.....	195,000,000	4,000,000	2.1	35,000,000	18.0	82,500,000	2,000,000	2.4	15,000,000	18.2
4.....	150,000,000	6,000,000	4.0	13,000,000	8.7	75,000,000	3,000,000	4.0	5,000,000	6.7
5.....	110,000,000	4,000,000	3.3	8,000,000	6.6	33,000,000	1,000,000	6.1	4,000,000	12.2
7.....	95,000,000	10,000,000	10.5	5,000,000	5.2	515,000,000	5,000,000	1.0	15,000,000	3.0
8.....	300,000,000	55,000,000	18.3	40,000,000	13.3	605,000,000	35,000,000	5.8	15,000,000	2.5
9.....	30,000,000	1,500,000	4.9	1,580,000	4.9	62,500,000	None.	0	1,000,000	1.6
10.....	59,000,000	1,500,000	2.5	1,500,000	2.5	780,000,000	Trace.	0	1,000,000	1.1
12.....	48,000,000	3,000,000	6.3	5,000,000	10.4	67,000,000	1,000,000	1.5	None.	0
15.....	843,500,000	25,000,000	10.2	22,500,000	9.3	41,500,000	None.	0	1,000,000	2.3
18.....	130,000,000	7,500,000	5.8	2,500,000	5.8	100,000,000	1,000,000	2.0	None.	0
21.....	119,500,000	1,000,000	1.9	2,000,000	1.9	40,000,000	None.	0	None.	0
Average.....			5.9		7.1			2.3		4.8

## COW MANURE

1.....	58,000,000	None.	0.0	750,000	1.3	51,000,000	800,000	1.6	2,000,000	3.9
2.....	73,250,000	7,500,000	10.3	5,000,000	6.8	41,000,000	4,000,000	2.4	2,000,000	4.8
3.....	497,500,000	10,000,000	2.0	30,000,000	6.1	295,000,000	25,000,000	8.5	12,000,000	4.2
4.....	91,000,000	2,000,000	2.1	7,500,000	8.1	68,000,000	2,000,000	2.9	3,000,000	4.4
5.....	102,500,000	3,500,000	3.4	2,000,000	1.9	44,250,000	2,000,000	4.5	3,500,000	7.9
7.....	36,250,000	1,000,000	2.8	None.	0	27,250,000	None.	0	1,000,000	4.0
8.....	43,000,000	2,000,000	4.6	None.	0	45,500,000	None.	0	1,000,000	2.2
9.....	17,500,000	None.	0	None.	0	37,000,000	None.	0	2,000,000	6.4
10.....	40,000,000	None.	0	1,000,000	2.5	36,500,000	500,000	1.4	5,000,000	14.0
12.....	96,000,000	3,500,000	3.7	4,500,000	4.7	49,500,000	1,000,000	2.0	5,000,000	10.0
15.....	187,000,000	7,500,000	4.0	4,000,000	2.1	53,000,000	1,500,000	2.8	1,000,000	1.9
18.....	75,000,000	10,000,000	13.3	2,000,000	2.7	125,000,000	10,000,000	8.0	2,000,000	1.6
21.....	64,000,000	1,500,000	2.3	5,000,000	7.8	40,000,000	Trace.	0	None.	0
Average.....			3.7		3.3			2.6		5.0

<sup>a</sup> *B. cereus* did not appear on the plates.

## AMMONIFICATION BY THE ORGANISMS IN QUESTION IN STERILIZED MANURED SOIL.

## SOIL INOCULATED WITH THE THREE ORGANISMS SEPARATELY

Table XII contains the data secured when the soil inoculated with pure cultures of each of the three organisms separately was subjected to the ammonification test previously described (p. 317). All of the organisms were found to be ammonifiers, and, so far as the individual organisms are concerned, the data indicates that, per organism, *B. cereus* is the most powerful ammonifier of the three. When the plate count of *B. cereus* showed 17,000,000 colonies per gram of soil on the tenth day after inoculation, the ammonia production was 22 mgm. per 100 gm. of soil (Table XII, Experiment II). On the other hand, a plate count of a flask inoculated with *Ps. fluorescens* showed 375,000,000 colonies per gram of soil on the tenth day and an ammonia production of 20.28 mgm. per 100 gm. of soil (Table XII, Experiment II); and a plate count from a flask inoculated with *Ps. caudatus* showed 220,000,000 colonies per gram of soil on the eighth day and an ammonia production of 17.84 mgm. per 100 gm. of soil (Table XII, Experiment II). This fact does not prove, however, that *B. cereus* is an important ammonifier in unsterilized manured soil. The data already discussed (p. 325) indicate that under natural conditions the organisms of the *B. cereus* group are present in manured soil in very small numbers and that the vegetative cells quickly disappear.

TABLE XII.—Ammonia produced by *B. cereus* and the non-spore formers inoculated separately into sterile manured soil

Experiment No. and time since inoculation	<i>B. cereus</i>				<i>Ps. caudatus</i>				<i>Ps. fluorescens</i>				
	Total count	Quantity of ammonia per 100 gm. of soil		Total count.	Inoculated flask	Blank control	Quantity of ammonia per 100 gm. of soil		Total count	Inoculated flask	Blank control	Quantity of ammonia (per 100 gm. of soil)	
		Mgm.	Mgm.				Mgm.	Mgm.				Mgm.	Mgm.
Experiment I:													
2 days.....	4,000,000	11.24	4.6	1,420,000,000	5.12	3.4	140,000,000	5.8	140,000,000	5.8	1.10		
4 days.....	5,000,000	10.92	.68	1,340,000,000	11.16	1.16	260,000,000	16.32	260,000,000	16.32	7.76		
9 days.....	10,000,000	12.16	1.16	1,480,000,000	20.4	7.76	115,000,000	11.8	115,000,000	11.8	2.72		
11 days.....	10,500,000	17.08	5.44	1,100,000,000	14.96	2.72	350,000,000	19.04					
Experiment II:													
2 days.....	8,000,000	7.12	3.04	114,000,000	7.48	4.4	105,000,000	18.76	105,000,000	18.76	4.34		
4 days.....	5,000,000	12.16	5.08	181,000,000	1.36	4.4	302,000,000	15.12	302,000,000	15.12	4.16		
6 days.....	22,500,000	10.2	3.4	194,000,000	10.16	5.44	305,000,000	20.00	300,000,000	20.00	6.64		
8 days.....	14,000,000	15.64	3.72	220,000,000	17.84	6.12	470,000,000	26.72	470,000,000	26.72	6.64		
10 days.....	17,000,000	22.00	6.24	205,000,000	17.48	5.74	375,000,000	20.28	375,000,000	20.28	6.64		
12 days.....	15,000,000	18.92	3.72	220,000,000	16.12	4.4	450,000,000	17.12	450,000,000	17.12	5.44		
14 days.....	21,000,000	21.68	5.08	260,000,000	16.64	4.4	150,000,000	14.28	470,000,000	14.28	6.60		
16 days.....	26,000,000	17.32	4.24	171,500,000	16.48	5.74							

## SOIL INOCULATED WITH A MIXTURE OF THE THREE ORGANISMS

Table XIII contains the data obtained as the result of inoculating sterilized manured soil with a mixture of the three organisms. In Experiment I all of the organisms were inoculated in approximately equal numbers. It is a noteworthy fact that while *Ps. fluorescens* and *Ps. caudatus* showed rapid development and were always present in large numbers, *B. cereus* never developed upon the plates and showed a very rapid decrease in the number of vegetative cells present in the smears examined under the microscope.

TABLE XIII.—Ammonia produced by a mixture of *B. cereus* and the two non-spore formers inoculated into sterile manured soil

Experiment No. and time since inoculation	Total count per gram of soil.			Quantity of ammonia per 100 gin. of soil.	
	<i>Ps. fluorescens.</i>	<i>Ps. caudatus.</i>	<i>B. cereus.</i>	Inoculated flask.	Blank control.
EXPERIMENT I:				Mgm.	Mgm.
0 days.....	<sup>a</sup> 2,573,000	<sup>a</sup> 2,406,000	<sup>a</sup> 2,380,000		
4 days.....	240,000,000	80,000,000	B. cereus did not appear on plates.	19.52	5.88
6 days.....	220,000,000	390,000,000		19.56	4.8
8 days.....	100,000,000	710,000,000		20.4	5.08
11 days.....	75,000,000	920,000,000		14.96	4.76
13 days.....	80,000,000	880,000,000		21.12	5.08
15 days.....	90,000,000	870,000,000		17.52	No test.
17 days.....	80,000,000	865,000,000		20.36	No test.
19 days.....	80,000,000	730,000,000		19.56	No test.
EXPERIMENT II:					
0 days.....	<sup>a</sup> 80,000	<sup>a</sup> 640,000	<sup>a</sup> 2,600,000		
7 days.....	250,000,000	1,190,000,000	B. cereus did not appear on plates.	15.96	7.12
9 days.....	130,000,000	1,020,000,000		18.2	7.48
15 days.....	80,000,000	860,000,000		21.16	5.92
23 days.....	140,000,000	590,000,000		21.12	6.12
27 days.....	195,000,000	420,000,000		19.52	6.12
30 days.....	50,000,000	330,000,000		17.36	6.32
31 days.....	60,000,000	290,000,000		15.96	6.12

<sup>a</sup> Computed from the number of organisms in the infusion used for inoculation.

Despite the very evident fact that *B. cereus* was not active in the samples tested, the amount of ammonia produced was quite marked. This was a strong indication that the ammonia produced was due to the activity of the only other organisms present, *Ps. fluorescens* and *Ps. caudatus*. In Experiment II the organisms were inoculated in the proportion of 1 individual of *Ps. fluorescens* to 8 of *Ps. caudatus* to 33 of *B. cereus*. Even with such a favorable start as this, *B. cereus* again failed to develop on the plates and showed a rapid decrease in the number of vegetative cells present. The degree of ammonification was marked also in this series.

Throughout the entire series of ammonification experiments a correlation seemed to exist between the time that had elapsed since the

inoculation of the soil and the amount of ammonia produced rather than between the number of organisms present and the ammonia production. The tests were continued until the apex of ammonia production was apparently reached. After this point had been reached, a steady decrease in ammonia production was noted, regardless of the number of organisms present. This was undoubtedly due to the depletion of available organic matter.

#### DISCUSSION OF RESULTS

The heterogeneous nature of soil, the great variety of organisms present, and the varying moisture content are all factors which make it practically impossible to carry on a study of ammonification in the soil under absolutely natural conditions. In order to control these things and to obtain comparable data, it is necessary to bring the soil into the laboratory for study. This introduces a difficulty, in that conditions governing the activities of organisms in the laboratory are generally at wide variance with conditions in the natural environment of these organisms. The reason for this variance is twofold: First, laboratory media may often be decidedly different from the soil in which the organisms are native; and second, the organisms in pure culture, as they are generally handled in the laboratory for purposes of control, do not behave as they would in competition or in association with the other organisms normally present in the soil. These artificial conditions must be kept in mind in considering the results obtained in the present work, but despite them there was one striking relation which invariably held true: the spore forming *B. cereus* never multiplied in manured soil with any degree of rapidity, while *Ps. fluorescens* and *Ps. caudatus* always did.

The data indicate that in soil where little organic matter is present and the processes of soil decomposition are practically at a standstill the spore former *B. cereus* occurs much more often than do the non-spore formers *Ps. fluorescens* and *Ps. caudatus*. When organic matter in the form of manure has been added to that same soil, however, and the processes of decomposition become active, the character of the flora changes entirely, and *Ps. fluorescens* and *Ps. caudatus* predominate over *B. cereus*. But the proof that these non-spore formers are the important ammonifiers in manured soil is decidedly difficult to secure.

As Conn (7, p. 254) has previously pointed out, there are four points which must be established before we can show conclusively that any particular chemical transformation in the soil is due to certain organisms:

- (1) The organism must be shown to be present in active form when the chemical transformation under investigation is taking place; (2) it must be shown to occur in larger numbers under such conditions than in the same soil in which the chemical change is not occurring; (3) it must be isolated from the soil and studied in pure culture; (4) the same chemical change must be produced by the organism in experimentally inoculated soil, making the test, if possible, in unsterilized soil. The fourth

requirement, however, can ordinarily be carried out only by inoculating sterilized soil, a procedure which does not give rigid proof, but which is fairly conclusive if carried out in connection with the other three requirements.

The data presented above offer fairly conclusive proof that these conditions have all been fulfilled by the organisms in question. The first step in the proof may be found in Tables IX, X, and XI, where it is shown that one or both of the non-spore-forming organisms are always present in active form in manured soil in which ammonification is occurring. That the second requirement is fulfilled is shown by Tables VIII, IX, X, and XI, in which it may be seen that the non-spore formers *Ps. fluorescens* and *Ps. caudatus* occur in much greater numbers in decomposing manured soil than in the same soil before the manure has been added, and that the spore former *B. cereus* occurs in great abundance in the soil before adding manure, but disappears almost entirely after manuring. The isolation of pure cultures, the third step in the proof, needs no comment, while the fourth is fulfilled, as seen by reference to Table XII, where it is shown that pure cultures of the organisms have the power of ammonifying manure in soil.

On the basis of the data obtained there are therefore no good reasons for believing that the spore-forming organisms play an important rôle in ammonifying manure in soil, and there is very good evidence that the non-spore formers *Ps. fluorescens* and *Ps. caudatus* are of primary importance in ammonification in manured soils.

## II.—TAXONOMIC STUDY OF TWO IMPORTANT SOIL AMMONIFIERS

By H. J. CONN

### DISTRIBUTION

The preceding section of this paper is concerned with the ammonifying powers of two soil bacteria, *Pseudomonas fluorescens* (Flügge) Migula and *Ps. caudatus* (Wright) Conn. Both of them are believed to be very widely distributed in nature. There is no question as to the wide distribution of *Ps. fluorescens*, because it has been described again and again by previous investigators as occurring in various localities. This, or some other similar organism, has been found most frequently in soil and in water; but has also been reported in air, butter, maple sap, and other substances. It has been observed by the present writer in practically all soils investigated, especially in soil that has been aerated or manured. *Ps. caudatus* is probably equally widely distributed; but the difficulty in recognizing it from published descriptions renders the literature concerning it of doubtful value. No references to similar organisms in soil have been found, but various writers have described yellow or orange liquefying bacteria found in water, some of which are undoubtedly the same as the organism studied here. Water was the source of Wright's *Bacillus caudatus*. The writer has observed it in water and in many soils, especially in freshly manured soil.

To aid in the identification by others of these two organisms studied by Bright, a detailed investigation of their physiology and cultural characteristics has been made, and the characteristics observed have been compared with those described by other writers. The following paper contains a description of these characteristics and a discussion of the probable relationships of these organisms in others.

### PSEUDOMONAS FLUORESCENS

*Pseudomonas fluorescens* (Flügge) Migula (1900, p. 889) was first described by Flügge (16, p. 289) as "*Bacillus fluorescens liquefaciens*." The description is rather meager, but the organism is plainly specified as a motile short rod, liquefying gelatin rapidly with the formation of a greenish-yellow fluorescence, producing a brownish growth on potato, and occurring in water and in decomposing material. This description indicates beyond question the group of fluorescent pseudomonads, even if the exact species or variety is uncertain. In the later edition of this book (Kruse, 26, p. 292) the organism is described more definitely with the following additional information: Size 0.3 to 0.5 by 1 to 2  $\mu$ ; no spores; Gram stain negative; optimum temperature 20° to 25° C. Kruse further



makes the statement that if all small deviations were designated as constant characters dozens of species must be established.<sup>1</sup>

The organism is much more fully described by Lehmann and Neumann (29, p. 272) under the name of *Bacterium fluorescens* (the adjective "liquefaciens" dropped to avoid a trinomial, and placed in the genus *Bacterium* because these authors placed only spore formers in *Bacillus*). They state that it is identical with *B. pyocyaneum* in all essential characters.<sup>2</sup> It is described as having polar flagella. *B. pyocyaneum* is described as producing no indol, hydrogen sulphid ( $H_2S$ ), nor gas from dextrose, but as converting nitrate into nitrogen; from which it is to be assumed that *B. fluorescens* agrees in these characteristics, although nothing definite is said on the subject except in regard to indol and hydrogen sulphid. Migula (38) placed this organism in his genus *Pseudomonas*, created to contain the rods with polar flagella. Migula describes *Ps. fluorescens* at some length, but lays greatest stress on cultural characteristics and adds little of importance to Kruse's description. Migula gives its diameter as about  $0.68 \mu$ .

Many other writers have described the same or some similar organism. Many different names have been given to fluorescent bacteria from time to time, Tanner (49) having recently stated that 95 different names had been found in a search through the literature. Many of these names are trinomials or worse, such as *Bacillus fluorescens putidus* Flüge (16), *B. fluorescens liquefaciens minutissimus* (Unna) Tommasoli (14); but others have conformed to approved rules of nomenclature. The greater number of the fluorescent organisms have been found in water, soil, or decaying organic matter; but one of the best known forms, the *pyocyaneus* type—more correctly named "*Ps. aeruginosa* (Schroeter) Migula"—causes blue pus. As mentioned by Kruse (26), there is great variation among these organisms, and if each variation be taken as a constant characteristic an almost endless variety of species could be named. This fact naturally raises the question how many of the names found in the literature are valid and how many are really synonyms, having been applied to mere physiological variations of a previously described species. Even the blue-pus organism is thought by some writers to be identical with the saprophytic forms. We have not yet sufficient data to straighten out completely the resulting confusion, but a careful search through the literature throws a little light on the matter. The information accumulated during the present work has made it possible to review this literature more intelligently than could have been done otherwise; and it seems well, therefore, to summarize the writings of others in regard to some of the more important fluorescent organisms.

<sup>1</sup> Wenn man alle kleinen Abweichungen als konstante Merkmale auffassen wollte, müsste man Dutzende von Arten aufstellen.

<sup>2</sup> Allen wesentlichen Eigenschaften.

## BACTERIUM TERMO

The name "*Bacterium termo* (Müller) Ehrb." was given by Ehrenberg to what he considered the *Monas termo* of O. F. Müller (34). The same name was used by various writers during the next three or four decades to designate almost any motile rod found abundantly in decaying organic matter. Finally Cohn (4, p. 196) described *B. termo* as a green fluorescent organism obtained from decomposing seeds by making a series of transfers into tubes of Cohn's solution.<sup>1</sup> By means of this same technic, a culture has been obtained agreeing fairly well with Cohn's organism, a vigorous denitrifier,<sup>2</sup> differing from all other fluorescent pseudomonads investigated here. Smith (47, p. 170), however, used this technic and obtained a green fluorescent organism differing distinctly (in failure to liquefy gelatin and in having but one instead of two flagella) from the one found in this laboratory. It seems doubtful, therefore, whether Cohn's organism actually represents one or several species; and as there is some question as to whether he was justified in his emendation of the species, the name is not used in the present bulletin.

Van Iterson (23) described a nonliquefying, fluorescent denitrifier (*B. denitrofluorescens*) which may perhaps be the same as Smith's "*B. termo*," or closely related to it. Other fluorescent, denitrifying bacteria have been described by Severin (45, 46) and by Jensen (24). It is evident, therefore, that in the group of fluorescent pseudomonads there are certain denitrifiers, one or more of which are especially adapted to growth in Cohn's solution. Severin and Jensen used the designation "*Bacillus pyocyaneus*" or "*Bacterium pyocyaneum*" for their fluorescent denitrifiers, so it is necessary to review the literature relating to the pyocyaneus type of organisms.

## PSEUDOMONAS AERUGINOSA

*Pseudomonas aeruginosa* (Schroeter) (44, p. 157) Migula (38, p. 884), or *Ps. pyocyanea* (Gessard, Flugge)<sup>3</sup> Migula (1900, p. 29), the blue-pus organism, has long been known, but there has been much confusion as to its name. Many writers have used the specific name "*pyocyaneus*," although others have recognized the priority of *aeruginosa*. *Bacillus*, *Bacterium*, and *Pseudomonas* have all three been used as the generic name, according to the generic definitions adopted by different authors. The name "*aeruginosa*" seems to be correct.<sup>4</sup>

<sup>1</sup> The formula of this solution is: Distilled water, 1,000 cc.; acid potassium phosphate ( $KH_2PO_4$ ), 1 gm.; magnesium sulphate ( $MgSO_4$ ), 5 gm.; neutral ammonium tartrate [ $(NH_4)_2C_4H_4O_6$ ], 10 gm.; potassium chlorid (KCl), 0.5 gm.

<sup>2</sup> The term "denitrification" in this paper is used strictly to refer to the liberation of free nitrogen from nitrate, not to the reduction of nitrate to nitrite or ammonia.

<sup>3</sup> Gessard (20) is generally quoted as the author of the term "*pyocyaneus*," although he did not employ it in accordance with strict taxonomic usage and apparently referred to an entirely different organism. The first correct use of the name *Bacillus pyocyaneus* for the true blue-pus organism was by Flugge (16, p. 265).

<sup>4</sup> The nomenclature of this organism is to be discussed in a paper by R. S. Breed and H. J. Conn, which is now in course of preparation, and will appear shortly in the *Journal of Bacteriology*.

Gessard (21) made a comparative study of this organism and some other fluorescent organisms. He concluded that it produces two pigments: a yellow-green, water-soluble pigment, and a blue-green, chloroform-soluble pigment, which he called "pyocyanin." He claims that it differs from *B. fluorescens liquefaciens* and *B. fluorescens putidus* (the nonliquefying type), as neither produces pyocyanin. Lehmann and Neumann (29, p. 272), however, claimed that the two organisms differ only in the intensity of the pigment, and remark concerning *B. pyocyaneum* that according to their conviction, this organism can not be sharply differentiated from *B. fluorescens*.<sup>1</sup> The opposite conclusion was reached two years later by Niederkorn (40), who studied a series of fluorescent cultures from various sources and decided that the *fluorescens* type and the *pyocyaneus* type are distinct, although each has numerous subvarieties. He states that the flagella of the *pyocyaneus* type are well defined,<sup>2</sup> but those of the *fluorescens* type are not; that the former takes the Gram stain more definitely than the latter; that the former grows best at 35° C., the latter at room temperature. The contrary opinion is expressed by Růžička (42, 43), who mentions these and other differences (except in regard to the Gram stain), but concludes that they are not constant. By cultivating the *fluorescens* type at 37° he obtains cultures of the *pyocyaneus* type; by growing the blue-pus organism in water, aerated with sterile air, he obtains cultures of the *fluorescens* type. Later Lehmann and Neumann (30, p. 411-413) continue the discussion, referring to the differences between the two types, laying considerable stress on the denitrifying power of the blue-pus organism, but repeating their earlier statement that one type grades imperceptibly into the other. They did not find either organism Gram-positive. Finally, Pribram and Pulay (41) made a study of the fluorescent group by serological methods and found it apparently to consist of several different species, *B. pyocyaneum* appearing distinct from *B. fluorescens*, although closely related to it.

The ability of the *pyocyaneus* type to convert nitrate into free nitrogen was apparently first mentioned by Lehmann and Neumann (29), who do not, however, mention the source from which the culture they studied was obtained. The following year, Weissenberg (51), apparently at the suggestion of Lehmann or Neumann, made a further investigation of *pyocyaneus* cultures from various sources, finding them all to be denitrifiers, while observing this ability with no organism of the *fluorescens* type. The same year Severin (45) wrote a paper on denitrifiers obtained from manure, one of which is fluorescent. This fluorescent culture he calls *B. pyocyaneus*, but does not show it to be the cause of blue pus.

One striking fact in this connection is that no one seems to have found a Gram-positive *pyocyaneus* culture which denitrifies or a Gram-negative

<sup>1</sup> Den Organismus scharf gegen *B. fluorescens* abzugrenzen, geht nach unserer Ueberzeugung nicht an.

<sup>2</sup> Wohl ausgeprägte.

one which does not denitrify. Those who report denitrification either state the organism to be Gram-negative (as do Lehmann and Neumann) or else make no statement in regard to the Gram stain. Those who have found it to be Gram-positive have not studied its action on nitrate. This suggests that two different organisms, one Gram-positive and pathogenic, the other Gram-negative, denitrifying, and probably saprophytic. If this be the case, the former is more likely to be distinct from the *fluorescens* type than the latter.

#### PSEUDOMONAS PUTIDA

*Pseudomonas putida* (Flügge) Migula (38, p. 912). The name "*Bacillus fluorescens putidus*"<sup>1</sup> was given by Flügge (16) to the nonliquefying, fluorescent type of organism. Eisenberg (14), besides this name, used the name "*B. fluorescens nonliquefaciens*" for what he considers a different organism, and in this is followed by Kruse (26) and Migula (38), the latter discarding the polynomial and renaming it "*Ps. Eisenbergi*." Lehmann and Neumann (30), however, do not consider it a distinct type and *Ps. putida* is the only nonliquefying species considered to day to have good standing.

Whether *Ps. putida* and *Ps. fluorescens* are distinct is also a question that is not entirely settled. Lehmann and Neumann do not question that they are distinct. Pribram and Pulay (41), as the result of their serological studies, conclude that they are not only distinct but that they stand the farthest apart of any of the fluorescent cultures studied. Edson and Carpenter (13), however, consider that there are so many gradations between rapid liquefiers and nonliquefiers that this characteristic can not be used to distinguish between species.

#### NUMBER OF FLUORESCENT BACTERIA

A summary of the literature, therefore, gives no satisfaction in deciding how many different pseudomonads possess the property of producing fluorescence in culture media. Some writers consider them all the same; others make two or three different species; still others believe there are several species; while, if we consider every name a distinct species, there are a hundred or more. A study of the literature, however, indicates that there are four or five types standing out more or less distinct from each other: (1) *Ps. aeruginosa*, the blue-pus organism, a Gram-positive, rapidly liquefying organism, producing the blue green pigment pyocyanin in addition to the fluorescent pigment, and possibly reducing nitrate to nitrogen. (2) *Ps. fluorescens*, a Gram-negative, rapidly liquefying saprophyte, showing poor growth or none at 37° C.,

<sup>1</sup> In the third edition of Flügge's book, Kruse (26) uses the name *B. fluorescens putidus*, evidently a misprint or mistake in spelling, because Flügge's description of the organism by the term "*stinkende*" shows plainly that "*putidus*" was the word he meant to use. Migula in renaming the organism follows Kruse's spelling, calling it *Ps. putida* (Flügge). Other writers, however, such as J. Eisenberg (14), Lehmann and Neumann (30), and Chester (2) have used the spelling "*putidus*."

and unable to convert nitrate to free nitrogen. (3) A Gram-negative, rapidly liquefying denitrifier, such as described by Lehmann and Neumann as *Bacterium pyocyaneum*. Whether these authors worked with the true blue-pus organism or not, there seems to be an organism of this description that is different from the true *Ps. aeruginosa*. Several such cultures have been isolated in this laboratory, all of which fail to show pyocyanin even when grown in nitrite broth (the method described by Eisenberg (15, p. 470) as showing the production of this pigment to advantage) and extracted with chloroform. These cultures have differed among themselves and may represent several varieties. Undoubtedly the *B. pyocyaneus* of Severin (45, 46), Jensen (24), and others, who studied denitrifiers from manure and soil, was an organism (or organisms) of this type rather than of the true *pyocyaneus* type. (4) A nonliquefying denitrifier described by Van Iterson (23) as *B. denitrofluorescens*, which is probably distinct from the above and from the following, although it has not been studied here. (5) *Ps. putida*, a nonliquefying organism unable to denitrify. Although some writers seem to think liquefaction an unsatisfactory basis for the separation of these species, there seems no chance for reasonable doubt that an organism unable to liquefy after six months is different from the very rapid liquefiers studied in the present work. The difficulty in making this distinction may perhaps be due to the failure to distinguish between true liquefaction by the living cells and slow digestion of the gelatin by enzymes liberated from the cells after death.

Further investigation is necessary before it can be decided whether these five types represent different varieties of the same species, five separate species, or even five different type species about which distinct groups of species (perhaps genera) should be gathered.

#### CHARACTERISTICS OF TYPICAL FLUORESCENT ORGANISMS

**MORPHOLOGY.**—Small, short rods, not much over  $0.5\ \mu$ , or perhaps somewhat smaller; no spores; a few flagella in a tuft at one pole; Gram-negative. (A few fluorescent spore formers have been described, and Edson and Carpenter (13) mention a weakly fluorescent peritrichic rod; but these are apparently unrelated organisms. The *pyocyaneus* type has been described as Gram-positive.)

**CULTURAL CHARACTERISTICS.**—Growth on agar smooth, soft to slimy; on potato smooth, brownish, medium discolored. Nearly all other cultural characteristics vary.

Greenish fluorescence is the most striking characteristic of the entire group, but it is not a constant characteristic. It is produced in some media and not in others. Gessard (21, 22), Lepiere (31), and Jordan (25) have studied the ability of these organisms to cause fluorescence with rather discordant results. They differ considerably in their conclusions as to the composition of the medium necessary for the produc-

tion of this pigment. The reason for this discrepancy may be in part, as suggested by Jordan, because of difficulty in obtaining absolutely pure chemicals; but it is undoubtedly also due to the varying behavior of different varieties. It has been observed in the course of the present work that two different strains may behave exactly the opposite, so far as concerns their ability to produce fluorescence in one or the other of some two media investigated. Any one strain, moreover, may vary considerably at different times in its ability to produce fluorescence. One particular strain has been studied in this laboratory which was thought to be *Ps. fluorescens* when first obtained from soil, although not showing fluorescence; but after having been cultivated for several generations on a beef-extract-peptone agar containing 0.1 per cent of nitrate, it gradually became fluorescent, and at the time of writing is one of the most strongly fluorescent cultures in this laboratory. (Another sub-strain of this organism, kept growing meanwhile on the same agar without nitrate, has developed no fluorescence.) A similar phenomenon was observed by Severin (45) upon cultivating a denitrifying strain in nitrate broth.

The strain used in Bright's experiments as reported in the first section of this paper was always found to cause decided fluorescence on all ordinary media.

RELATION TO OXYGEN.—Apparently all of the group are strictly aerobic. This is certainly true of all that have been studied here.

LIQUEFACTION OF GELATIN.—Typical *Ps. fluorescens* is a very vigorous liquefier. Slow liquefiers are common, as shown by Edson and Carpenter (13), although but few have been found in the present work. Non liquefiers have been observed occasionally in the soils investigated here.

The gelatin colonies of fluorescent organisms vary according to the rapidity of liquefaction. Typical *Ps. fluorescens* produces a rapidly liquefying colony with entire edges that liquefies the entire plate in a few days. The strain studied in this work produced a colony of this type, also characterized by its clear structureless center; fluorescence was sometimes present, sometimes absent.

AMMONIFICATION.—Ammonia is produced from proteid by all the fluorescent organisms so far as they have been studied. Blanchetière (1) has made a careful study of the ammonification of asparagin by a fluorescent liquefier, agreeing well with the strain used in the present work; and has found that it easily converts the amid nitrogen of this compound into ammonia, but the aspartic nitrogen less readily.

ACTION ON SUGARS AND GLYCERIN.—Apparently no fluorescent organism has been recorded as producing gas from sugars or glycerin. Nearly all writers have found acid to be produced from dextrose, but in regard to other sugars the results are conflicting. The reason for this in part is that the technic generally used is bound to give meaningless results. Thus, Tanner (49) and Edson and Carpenter (13) both determine acid

production by titration, the latter writers titrating hot, a procedure which Clark (3) has shown to be illogical. Tanner finds acid production only in dextrose, while Edson and Carpenter find it common with the other sugars, undoubtedly because the H-ion concentration is increased by heating the culture previous to titrating.

Blanchetière (1) avoids this error by using litmus agar. He finds acid produced from dextrose and levulose, but not from the disaccharids; but as levulose is a difficult sugar to purify, and as Blanchetière says nothing about the source of his sample, he leaves some doubt in the reader's mind as to whether it was actually free from dextrose. He distinctly states that lack of acid reaction in this medium does not mean failure to produce acid, but simply that not enough acid is produced to neutralize the ammonia formed from the peptone. This shows Blanchetière realizes another source of error, but feels unable to overcome it. Plainly, with these two sources of error, the data in the literature as to acid production by fluorescent organism are not reliable.

In the present work Blanchetière's technic has been modified by using bromcresol purple in place of litmus as an indicator. The result has been in practically every case to find acid produced from dextrose and sucrose, but not from lactose and glycerin. The strain studied in Bright's experiments, above reported, showed these reactions very constantly. A synthetic medium containing ammonium tartrate as its only source of nitrogen<sup>1</sup> was then used in an attempt to overcome the error resulting from the presence of peptone in ordinary agar, and somewhat different results were obtained. Even with this method there was no agreement in the results obtained with the different fluorescent organisms. The strain used by Bright showed acidity from dextrose and sucrose, the latter reaction disappearing after the first day; while another strain agreeing with it in every respect showed strong and persistent acidity in sucrose as well as dextrose. The conclusion was reached that *Ps. fluorescens* produces acid from both dextrose and sucrose, but that with the latter sugar the acid production is likely to be obscured by other activities tending to lower the reaction of the medium.

**REDUCTION OF NITRATE.**—The literature is full of conflicting data in regard to the action of fluorescent bacteria on nitrate. There are several different possibilities: (1) Reduction to nitrite; (2) reduction to nitrite, then to ammonia; (3) reduction to ammonia without appreciable accumulation of nitrite; (4) reduction to free nitrogen—namely, denitrification; (5) assimilation of the nitrogen of the nitrate, with or without previous reduction. It has not proved possible to devise a simple test to distinguish with certainty between these five different possibilities, hence, the confusion.

<sup>1</sup> The formula of this medium was: Distilled water, 1,000 cc.; agar, 15 gm.; calcium chlorid ( $\text{CaCl}_2$ ), 0.5 gm.; potassium phosphate ( $\text{K}_2\text{HPO}_4$ ), 0.5 gm.; ammonium tartrate [ $(\text{NH}_4)_2\text{C}_4\text{H}_6\text{O}_8$ ], 10 gm.; with 10 gm. of the sugar (or glycerin) under investigation.

Conversion into free nitrogen is the easiest to determine. It has already been seen that fluorescent denitrifiers have been described in the past. Here, they prove to be common enough in soil to be obtained frequently from ordinary soil culture plates. Neither Edson and Carpenter (13) nor Tanner (19) found any among the various cultures they studied; but they both used nitrate broth containing only 0.1 per cent of peptone, in which appreciable gas production has never been observed here. Most vigorous gas production has been observed in broth or agar containing 1 per cent of peptone. Typical *Ps. fluorescens*, however, has never been found to convert nitrate into nitrogen.

Conversion into ammonia is ordinarily impossible to demonstrate by any simple test, because ammonia can be produced from any nitrogenous substance, and some organic nitrogen is ordinarily present in media. Conversion into nitrite is easy to demonstrate, provided the organism investigated does not convert the nitrite into ammonia or assimilate it as fast as produced. *Ps. fluorescens* is generally considered to produce nitrite, but Franzen and Lohmann (18) studied two strains of what were presumably *Ps. fluorescens* without observing any action at all on the nitrate. Certain strains of fluorescent liquefiers have been studied here which produce no appreciable amount of nitrite in media containing peptone or ammonium chlorid, but produce considerable nitrite in an agar containing no nitrogen except potassium nitrate.<sup>1</sup> One strain has been found which does not produce nitrite (nor ammonia) even on the latter medium. This suggests that some strains of *Ps. fluorescens* lack the ability to produce nitrate, wholly or in part, and never attack the nitrate in the presence of more readily available nitrogen. This may explain Franzen and Lohmann's findings (18).

The question naturally arises whether those organisms that produce no nitrate in ordinary nitrate media constitute a different species from typical *Ps. fluorescens*. So far as tested, these differences between the strains have proved constant. Nevertheless, the different strains agree in all other particulars investigated, and the data at hand are not considered to warrant the establishment of separate species. As typical *Ps. fluorescens* is generally considered to produce nitrite in nitrate broth the strain selected for Bright's work in the preceding section was one showing considerable nitrite on all the nitrate media investigated.

**DIASTATIC ACTION ON STARCH.**—This test was made by the method of Allen,<sup>2</sup> streaking the cultures over a plate of agar containing soluble starch, and flooding with iodine after seven days. In general no digestion of the starch was observed, although some of the cultures seemed to show a very narrow zone around the growth where the starch had disappeared.

<sup>1</sup> The formula of this medium was: Distilled water 1,000 cc., agar 15 gm., calcium chlorid ( $\text{CaCl}_2$ ) 0.5 gm., potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) 0.5 gm., potassium nitrate ( $\text{KNO}_3$ ) 1 gm., dextrose or sucrose 10 gm.

<sup>2</sup> ALLEN, PAUL W. A SIMPLE METHOD FOR THE CLASSIFICATION OF BACTERIA AS TO DIASTATIC PRODUCTION. *Jn Jour. Bact.*, v. 3, no. 1, p. 11-17, illus. 1915.



ACTION ON MILK.—Digestion without previous coagulation.

PRODUCTION OF INDOL.—Statements in the literature are discordant. A number of different strains of *Ps. fluorescens* have been tested here for indol production, a feeble or moderate reaction having been obtained. The test is not considered to have much significance.

#### BRIEF SUMMARY OF CHARACTERISTICS OF TYPICAL PSEUDOMONAS FLUORESCENS

In the following summary the characteristics written within parentheses apply to typical cultures only (including the strain studied by Bright); the other characteristics apply not only to typical cultures but to all the cultures studied of the *fluorescens* type—(that is, type 2, p. 337).

MORPHOLOGY: Short, lophotrichic, Gram-negative rods about  $0.6 \mu$  in diameter. No spores.

GROWTH ON AGAR: Soft, smooth, with greenish fluorescence if conditions are favorable.

GELATIN COLONIES: (Large), liquefied (center structureless), edges entire.

RELATION TO OXYGEN: Strictly aerobic.

AMMONIA produced from organic nitrogenous matter.

ACID PRODUCTION from dextrose and sucrose, but not from lactose or glycerin.

NITRATES reduced to nitrite (in peptone media containing no nitrogen except the nitrate).

DIASTATIC ACTION ON STARCH: Weak or none.

MILK digested without coagulation.

#### PSEUDOMONAS CAUDATUS

*Pseudomonas caudatus* (Wright), (53, p. 444) Conn has been recognized by the writer for a number of years and has been mentioned in previous publications (5, 7-11), but not named. It is now believed to be identical with *Bacillus caudatus* Wright. Earlier surveys of water bacteria by Frankland (17), Tils (50), and Zimmerman (54) contain descriptions of orange or yellow liquefying bacteria, but they are either meagerly described or else show marked differences from the organism studied here. The identification with Wright's organism is based primarily upon a color plate showing the gelatin colony and upon his description of the morphology. He describes the morphology of the organism as follows (53, p. 444):

A rather small, slender, nonmotile bacillus, with conical ends, occurring often in pairs and in longer forms, sometimes threadlike, which may show irregular segmentation; no spore formation observed.

His illustration of the colony agrees well with the present organism as to structure and agrees as nearly with the shade of orange observed as could be expected in a colored plate 20 years old. Although Wright does not give the size of the organism in exact figures and calls it immotile, there is little question as to its identity.

MORPHOLOGY.—Ordinarily the organism is a very slender rod, so small that its diameter is difficult to measure with the ordinary microscope.

It is about  $0.2\ \mu$  in diameter. Its length is ordinarily about  $2\ \mu$ , but, as stated by Wright, longer forms occur. These rods stain solid with the ordinary bacterial stains, such as fuchsin or methylene blue; but with the more delicate dye, rose Bengal, they appear to be made up of tiny granules. Cultures a few days old are sometimes made up wholly of these granules, each about  $0.2\ \mu$  in diameter. Such a preparation looks like a very tiny micrococcus. Cultures of this sort have proved to be alive upon transfer to fresh media, but whether the granules are capable of growth or whether the multiplication is carried on by a few stray rods present in too small numbers to be observed under the microscope is still an unanswered question. This suggests very strongly Löhnis and Smith's idea (35) as to life cycles among bacteria, but as yet it has not proved possible to find whether that is the true explanation of this case. The granules may be degenerate forms, a possibility suggested by the rapidity with which cultures die, or the organism may be actually a coccus that has a tendency to produce short chains or filaments in young cultures.

The majority of the cultures show no motility, although occasionally one is observed that is distinctly motile. This undoubtedly explains why Wright (53) called the organism immotile. An idea of the difficulty in studying motility can be obtained from the trouble encountered in demonstrating flagella on the strain used in Bright's work. This strain was kept under observation for a few months without observing any motility, when at last, quite unexpectedly, a distinctly motile culture of it was obtained. On the same day two other strains, previously showing no motility, were found to be motile. No apparent reason could be found for this sudden development of motility, which persisted through at least three or four generations. Meanwhile flagella preparations were made from the strain used by Bright in the work reported above, and one or two organisms were observed with a single flagellum each. This flagellum is rather long in comparison to the length of the rod. This finding agrees with previous studies of this organism made by the writer. Three strains in all have been successfully stained, and about 10 different organisms have been observed with a single polar flagellum each. Preparations were always too poor to allow photomicrographs, but there seems to be sufficient evidence to establish the presence of one polar flagellum. For this reason Wright's name, "*Bacillus caudatus*," is changed to *Pseudomonas caudatus*.

CHROMOGENESIS.—Next to its morphology, pigment production is the most striking characteristic of *P. caudatus*. The pigment grades from yellow to orange. On potato and gelatin it is generally distinctly orange, while on beef-extract peptone agar it is more of a yellow. Its color on the latter medium is practically the same as that which is typical of the orange pyogenic cocci, designated cadmium-orange by Winslow and Winslow (52) in their book on the Coccaceae. The typical color, indeed, is exactly the shade of cadmium orange which the Winslows

found most common among the orange cocci. One strain has been found which was typical in color upon isolation from soil, but which lost its chromogenesis upon cultivation, not regaining it even after cultivating for a while in sterilized soil. This strain retained its typical morphology and differed from the other cultures at first in no other respect except that it was unable to digest soluble starch. Later it was found to have lost its power of producing nitrite upon nitrate-peptone media. No data are at hand to show whether or not it digested starch before it lost its pigment-producing power. The change in the color of this culture can hardly have been due to an impurity, because three separate sub-strains of this one strain all lost their pigment-producing power at exactly the same time. This shows that chromogenesis, striking as it is in typical cultures, is not an absolutely constant characteristic.

PHYSIOLOGY.—Perhaps the most striking physiological peculiarity of the organism is the difficulty of cultivating it under laboratory conditions. The only way found to keep it vigorous is by transfers every few days onto agar that has been freshly melted and solidified so as to have considerable water of condensation on its surface. This fact is unfortunate, for it makes it practically impossible to keep stock cultures of the organism for purposes of comparison with cultures of other investigators.

RELATION TO OXYGEN.—The organism is very strictly aerobic. In fact, it grows poorly in liquid media, even in an open test tube.

LIQUEFACTION OF GELATIN.—All cultures liquefy gelatin. The rapidity of liquefaction varies, although in general it is quite rapid.

Gelatin colonies usually liquefy to a diameter of about 1 cm. in four days. Liquefaction is most rapid on the plates made directly from soil, old cultures liquefying more slowly. The colonies have typically a radiate structure, although the typical structure is observed only immediately after isolation from soil. Edges of colonies are entire.

AMMONIA PRODUCTION.—As shown by Bright in the preceding paper, *Ps. caudatus* is a vigorous ammonifier.

ACTION ON SUGARS AND GLYCERIN.—In the early work with this organism (Conn, 5, 10) tests for acid production were made in sugar broth as recommended in the report of the committee of water analysis of the American Public Health Association.<sup>1</sup> Very irregular results were obtained and in mentioning this type (5, p. 103) question marks were placed over those figures in the group number referring to the dextrose, sucrose, and glycerin, although at that time no evidence at all of acid production in lactose had been obtained. Later (10, p. 8) it was thought that this irregularity must be due to poor growth in liquid media, so the recent tests have been made in sugar agar containing some indicator. The most satisfactory indicator has proved to be bromocresol purple. Using standard agar in this work, the writer divided the strains studied

<sup>1</sup> American Public Health Association. Standards methods for the examination of water and sewage, ed. 2, p. 127-128. New York, 1912.

into two groups, one<sup>1</sup> producing no acid and the other (containing the majority of the strains) producing acid from dextrose and sucrose but not from lactose or glycerin. There proved to be some irregularity upon repetition of the test, but not a great deal. It was then felt that the difference between these two groups of strains might be that one produced more alkalinity from the peptone than the other and that its acid production was thus obscured. The test was therefore repeated a few times on a tartrate agar<sup>2</sup> in which *Ps. caudatus* was found to cause no change in reaction unless some sugar were present. With this medium more consistency was observed upon repetition of the test, but the difference between the two groups was still sharp. The acid group acidified lactose in this medium as well as dextrose and sucrose. It is therefore concluded that typical *Ps. caudatus* produces acid from dextrose, sucrose, and lactose, but not from glycerin, its acid production from lactose being too weak to neutralize the alkalinity produced from the peptone if growing in ordinary media. The nonacid strains, with the exception of the nonchromogenic one, died while under cultivation in the laboratory; so it is felt that their failure to produce acid may have been the first evidence of loss of vigor. Hence, they are not considered to be distinct from the typical acid formers. The strain used by Bright in the experiments reported above was a vigorous acid producer.

NITRATE REDUCTION.—Irregular results were obtained with this test also. Ordinary nitrate broth proved so unsatisfactory that tests were made on agar slants as described for *Ps. fluorescens* (p. 341). On beef extract peptone agar, the acid group of strains, above mentioned, showed a strong nitrite reaction; the nonacid group, with the exception of the nonchromogenic strain, showed no nitrite; the nonchromogenic strain when first tested was distinctly nitrite-positive, but after a few months all three substrains of this organism were found to have lost their nitrite-producing power. To investigate this matter further, the synthetic sucrose-nitrite agar<sup>3</sup> already used for *Ps. fluorescens* was employed. On this agar an occasional culture was found to produce nitrite that had showed no nitrite reaction on the nitrate-peptone agar, and ammonia was observed in almost all cases. Growth was very poor, however, with the nonacid group of strains. Inasmuch as there was no possible source of ammonia in this medium except the nitrate, the conclusion was drawn that *Ps. caudatus* reduces nitrate to nitrite and ammonia, but that some cultures convert the nitrite into ammonia so rapidly that a nitrite test is generally negative. The presence or absence of the nitrite test depends upon the relative rate of these two processes, which is probably associated with the vigor of the culture. Hence, the failure of the nitrite test is no proof that any particular culture is not *Ps. caudatus*.

<sup>1</sup> One of the strains in this group was the one that had lost its pigment-producing power.

<sup>2</sup> Formula given in footnote, p. 340.

<sup>3</sup> Formula given in footnote, p. 341. Sucrose (not dextrose) was used in this formula because of the presence of ammoniacal impurities in the dextrose on hand.

DIASTATIC ACTION ON STARCH.—This test was made by the method of Allen.<sup>1</sup> All the cultures of *Ps. caudatus* studied, except the nonchromogenic strain, gave a strong reaction, but the nonchromogenic strain showed no digestion of the starch. These results were the same upon frequent repetition of the test.

ACTION ON MILK.—No change in appearance or reaction.

PRODUCTION OF INDOL.—The results of this test have generally been negative, although a few cultures have shown a weak reaction. It is not impossible that they would all produce indol if tested under conditions favorable to the growth of this organism; but the test has always been made in liquid media (Dunham's solution), and as yet no effort has been made to improve the technic.

#### BRIEF SUMMARY OF CHARACTERISTICS OF TYPICAL PSEUDOMONAS CAUDATUS

In the following summary the characteristics written within parentheses apply to typical cultures only (including the strain studied by Bright); the other characteristics apply to all the strains studied:

MORPHOLOGY: Long, slender, granular, Gram-negative rods, about  $0.2\ \mu$  in diameter, with a single polar flagellum. No spores. Old cultures often appear like cocci,  $0.2$  to  $0.4\ \mu$  in diameter.

GROWTH ON AGAR: Soft, smooth (cadmium orange).

GELATIN COLONIES: Small (to medium sized)—i. e., up to 1 cm. in diameter (orange, structure radiate), edge entire.

RELATION TO OXYGEN: Strictly aerobic.

AMMONIA produced from organic nitrogenous matter.

ACID PRODUCTION: (from dextrose, sucrose, and lactose) but not from glycerin.

NITRATES reduced to nitrate and ammonia (with accumulation of nitrite).

DIASTATIC ACTION ON STARCH: (strong).

MILK unchanged.

#### SUMMARY

(1) The statement recently made by one of the authors that non-spore-forming bacteria are most active in manured soil has been verified. This is contrary to the generally accepted idea that spore-forming bacteria are the important ammonifiers in soil.

(2) Of these non-spore-forming organisms that are especially active in manured soil, two of the most easily recognized are *Pseudomonas fluorescens* (Flügge) Migula and *Ps. caudatus* (Wright) Conn. They have therefore been selected for special study.

(3) Pure cultures of *Ps. fluorescens* and *Ps. caudatus* multiply much more rapidly in sterilized manured soil than do pure cultures of *Bacillus cereus* Frankland (selected as a typical spore former).

(4) When sterilized manured soil is inoculated with a mixture of these three organisms in pure culture, the two non-spore formers immediately gain the ascendancy, *B. cereus* occurring in too small numbers for detection by the ordinary methods of study.

<sup>1</sup> ALLEN, PAUL W. OP. CIT., 1913.

(5) In field soil to which there has been no addition of organic matter for several years, *Ps. fluorescens* and *Ps. caudatus* were rarely found, while *B. cereus* was a common organism.

(6) When this same soil was mixed with manure and potted, *Ps. fluorescens* and *Ps. caudatus* immediately multiplied rapidly, while but small numbers of *B. cereus* spores and no active forms of *B. cereus* could be found.

(7) All three of these organisms are vigorous ammonifiers when tested in pure culture.

(8) The activity of the non-spore formers and the absence of activity of the spore formers in unsterilized manured soil leads to the conclusion that *Ps. fluorescens* and *Ps. caudatus* are important ammonifiers of manure in soil, while there is no evidence that *B. cereus* takes part in this process.

(9) Detailed descriptions are given of the two ammonifying organisms studied.

(10) The culture of *Ps. fluorescens* studied has been compared with other fluorescent bacteria isolated from soil, and a review of the literature relating to fluorescent bacteria has been made. It has not proved possible to fix definite limits for this species.

(11) *Ps. caudatus* is the name now assigned to the organism previously denoted by one of the writers as the "orange-liquefying type." It is apparently identical with the organism described by Wright in 1895 (54), and seems to be quite common in soil and water.

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